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(54) Title: FUNCTIONAL SURROGATES OF ANALYTES OF INTEREST AND METHODS OF OBTAINING AND USING SAME

(57) Abstract

Functional surrogates are disclosed which serve as mimics of naturally occurring molecules, such as analytes of interest present in a given sample. In particular, functional surrogates (including epitopes and mimetopes) of macromolecular moieties, including large to medium-sized proteins, are described. The functional surrogates of the present invention are useful in a variety of applications, including diagnostic, prophylactic, and therapeutic applications. Indeed, where the detection of a macromolecular moiety is hampered by its size, a functional surrogate of the present invention, serving as the mimic of the macromolecular moiety, may be better suited for a given diagnostic assay. Methods of obtaining functional surrogates, various methods of their use, and compositions, including kits, are also described. Accordingly, certain binding peptides, including those of a well defined sequence, have been discovered, which can be used in a number of affinity assays, including those utilizing fluorescence polarization immunoassay (FPIA), enzyme multiplied immunoassay techniques (EMIT) or cloned enzyme donor immunoassays (CEDIA), to name a few.

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FUNCTIONAL SURROGATES OF ANALYTES OF INTEREST AND METHODS OF OBTAINING AND USING SAME

1. Field of the Invention

The present invention relates to functional molecular surrogates of naturally occurring molecules, including a variety of analytes of interest, such as large to medium-sized proteins. The functional surrogates of the present invention can be used in a variety of applications, including diagnostic, prophylactic, and therapeutic applications. In particular, large macromolecular moieties whose detection may be impractical under certain assay conditions, such as the conditions of homogenous immunoassay techniques, are detected successfully with the aid of the functional surrogates of the present invention. Methods of obtaining functional surrogates, various methods of their use, and compositions, including kits, are also described. The invention also relates to certain constructs comprising DNA sequences encoding selected functional surrogates that exhibit the affinity and/or related characteristics required to mimic the function and/or behavior of the naturally occurring analyte molecules, transforming vectors including the constructs, in addition to bacteriophage and microorganisms harboring same.

2. Background of the Invention

The detection of various analytes that may be present in a given sample has always been of principal interest in science and medicine. The need for a method of determining the presence or absence of a given analyte of interest is particularly acute in a clinical

setting, where assay conditions can be less than ideal, tensions especially high, and where speedy, reliable techniques may make the difference in the success or failure of the clinical treatment.

In most clinical settings, the assays for the detection of analytes of interest are indirect or of a heterogenous nature. Such heterogenous assays are time consuming and often require labeled antibodies for binding detection, not to mention solid carriers for use in separating bound from unbound antigenic species. Nonetheless, enzyme immunoassays (EIA) techniques are widely used for analyte detection because they are frequently the most effective of the available methods, or they may be the only method available for measuring the particular analyte of interest. See, for example, Porstmann, T. and Kiessig, S. T., in *J. Immunol. Meth.* (1992) 150:5-21, for a discussion of basic EIA techniques, including unlabeled (based on secondary immune reactions, such as precipitation and agglutination) and labeled (divided between so-called 'reagent-observed' and 'analyte-observed') methods. For the determination of both haptens and high molecular weight substances, the authors favor the labeled method, which they characterize as using monoclonal antibodies, as being of greater sensitivity, larger measuring range, and lower susceptibility to disturbing influences.

Despite the great success enjoyed by enzyme immunoassays, artifacts and limitations persist. In particular, differences between the results of solution versus solid-phase techniques have been shown. See, e.g., Pesce, A. J. and Michael, J. B., in *J. Immunol. Meth.* (1992) 150:111-119. These differences are due to a number of factors such as surface phenomena, changes in molecular structure on binding to a surface, changes in the valence of antibodies and antigens, and steric

constraints. For other limitations of EIA, including sources of interference, the reader is referred to the article by Pesce and Michael, *supra*.

2.1. "Homogenous" Affinity Assays

"Homogenous" enzyme immunoassays, those not requiring a surface bound component or a wash step, have been in use for a number of years since Rubenstein and co-workers described the inhibition of lysozyme activity on addition of morphine antibodies to a conjugate of morphine and lysozyme. Rubenstein, K. E., Schneider, R. S., and Ullman, E. F. *Biochem. Biophys. Res. Commun.* (1972) 47(4):846-851; U.S. Patent No. 4,190,496. When these workers discovered that the addition of free morphine reduced the inhibition of enzyme activity in proportion to the amount of free morphine added, the "homogenous" EIA technique was born.

The extension of the technique was later shown for the detection of other haptens, including drugs and hormones, and to the use of other enzymes, such as malate dehydrogenase, glucose-6-phosphate dehydrogenase (G6DPH), amylase, and beta-galactosidase. Gibbons, I. et al. *Anal. Biochem.* (1980) 102:167-170. The extension of the technique to macromolecular antigens proved more difficult, however, and such assays were adversely affected by serum. Moreover, the intimate interaction between enzyme and bound antibody, which is responsible for the conformational effects that give rise to the inhibition of enzyme activity, is less intimate and in fact attenuated when the enzyme is bound to a large protein antigen. Indeed, binding to the large protein antigen may sterically inhibit the enzyme to begin with and prevent the enzyme from interacting with

its substrate. Some success for the detection of human IgG was professed. See, Gibbons, I. et al., *supra*. Still others have tried to improve the sensitivity of homogenous EIA for the detection of macromolecular antigens by the use of modified labels, such as fluorogenic substrates for the enzyme. Armenta, R. et al., in *Anal. Biochem.* (1985) 146:211-219, describe an assay for serum ferritin using a beta-galactosidase-ferritin conjugate and dextran-linked beta-galactosyl-umbelliferone as enzyme substrate. A 1000-fold increase in sensitivity in going from a chromogenic substrate to a fluorogenic substrate was asserted. However, serum interference remained problematic due to the presence of antibody against beta-galactosidase in the patient samples.

2.2. Automated Assays

In recent years the major trend in the field has been toward non-isotopic assays capable of being automated. See, for example, Gosling, J.P., in *Clin. Chem.* (1990) 36(8):1408-1427. That is, immunoassays can be run manually by technicians performing the reagent addition steps - "manual assays", or on automated instruments - "automated assays". Automated assays can be run on either dedicated immunoassay instruments or on existing clinical chemistry analyzers. Dedicated Immunoassay instruments are usually differentiated by the detection mode used to monitor the assay (e.g., chemiluminescence, fluorescence, particle counting) and the method used, as in the case for heterogeneous assay systems, to separate free and antibody bound labeled ligand. Additionally a dedicated instrument is limited in that it will only run assays formulated specifically for that technology and particular detection system.

Conversely, homogenous immunoassays, without need for a separation or wash step, are particularly well suited for running in a conventional automated clinical chemistry analyzer. See, Khanna, in *Principles and Practice of Immunoassay*, C.P. Price & D.J. Newman (Eds.), Stockton Press, New York (1991) pp. 326-364.

The Enzyme Multiplied Immunoassay Technique (EMIT), popularized by Rubenstein et al., *supra*, can be run automatically on such clinical chemistry analyzers. As noted above, EMIT is a competitive homogeneous EIA in which an analyte is labeled with enzyme (most commonly a hapten conjugated to G6PDH). Binding of antibody to hapten G6PDH results in a decrease of G6PDH activity. A competition is set up between labeled and unlabeled hapten for a limited number of antibody binding sites. Increased amounts of hapten in the sample lead to less antibody available to bind to the labeled hapten, hence increased G6PDH activity is the result of increased concentration of hapten in the sample. The assay reagents readily lend themselves to being run on automated clinical chemistry analyzers and require only rate measurements at 340 nm for monitoring. Enzyme activity is monitored by measuring the rate of NADH formation at 340 nm; i.e., the assay only requires a regular UV detection system for measurement.

There has been no commercialized application of EMIT for the measurement of large analytes (e.g., proteins and other macromolecular moieties) because of the lack of a suitable G6PDH-macromolecule conjugate whose activity can be inhibited; that is, the conjugation of G6PDH to a large molecule will inherently render G6PDH inactive.

As stated previously, in an EMIT assay, binding of exogenous

antibody to enzyme-labeled antigen results in a change (a decrease) in observed enzymatic activity. Labeled and unlabeled antigen compete for a limited number of antibody binding sites. Hence, the concentration of antigen in the sample is directly proportional to the concentration of free labeled antigen. Accordingly, the greater the concentration of antigen in the sample, the greater the observed enzyme activity.

The only commercialized EMIT assays have been for the measurement of haptenic molecules, such as drugs of abuse or therapeutic drugs. Efforts, such as those by Gibbons, I., et al., *supra*, and Armenta, R., et al., *supra*, to extend the assay to macromolecular antigens have met with limited success. Both efforts require reagent incubation times in the order of hours, suffer from serum interferences and, in the case of the ferritin assay, require a detector for measuring fluorescence. In other words these assays were substantially inferior in ease of use and performance. It follows that in terms of ease of use, the EMIT procedure would only be practical for assays of haptens - small molecules such as drugs, as larger molecules, such as polypeptides or proteins, would inherently inactivate the enzyme activity on conjugation to the G6PDH.

In addition, an attempt to generalize this technology to other proteins of commercial importance is limited by the fact that in a competitive assay format, substantial amounts of highly purified analyte are required for conjugation to the enzyme. For many proteins, this requirement is prohibitive.

2.3. Other "Homogeneous" Affinity Assays

Other homogeneous enzyme immunoassays have been

described. For example, Jenkins, S. H., in *J. Immunol. Meth.* (1992) 150:91-97, discusses in addition to EMIT, substrate-labeled fluorescence immunoassay (SLFIA), prosthetic group-labeled immunoassay (PGLIA) or apoenzyme reactivation immunoassay (ARIA), cofactor-labeled immunoassay, inhibitor-labeled immunoassay, and cloned enzyme donor immunoassay (CEDIA). All these techniques are susceptible to interferences present in the sample, however, as there is no wash step. The search for ways to measure large analytes continues.

In the FETI technique, fluorescence excitation transfer immunoassay, the assay can be done in several configurations. The general principle is that two members of a binding pair are labeled, one with a fluorescein analog, the other with a rhodamine analog. The mixture is excited at the fluorescein absorption wavelength. If the labeled constituents are bound to each other, an energy transfer can take place and the fluorescein emission quenches the rhodamine excitation. This phenomenon permits an index of binding to be measured. In a relevant assay configuration, two distinct monoclonals are labeled. Energy transfer occurs only when the labels are brought into proximity by binding to the analyte. A fluorimetric analyzer has been designed and built to run a panel of FETI assays along with EMIT small molecule assays (reading NADH fluorescence). See, Ullman, E.F., Schwarzberg, M., Rubenstein, K.E., in *J. Biol. Chem.* (1976) 251(14):4172-8; Ullman, E.F., Khanna, P.Y., in *Methods in Enzymology* (1981) 74:28-60.

Another major assay is enzyme channeling. The concept is to label each of two monoclonal (or polyclonal) antibodies with a different enzyme. The two enzyme labels are coupled in the sense

that the product of one is a substrate for the second. In a specific instance, one antibody is labeled with glucose oxidase (GO). The second is labeled with horseradish peroxidase (HRP). The peroxide produced in the GO reaction is reduced by the HRP, resulting in oxidation of a leuco dye and production of a color. The coupled reactions go much faster when the two enzymes are held in proximity as when the antibodies to which they are attached form a complex with an antigen. The rate of color production is thus an index of analyte concentration. The principle could be demonstrated but has never worked well as the magnitude of the channeling effect is simply too small. See, Gibbons, I., et al., in *Methods in Enzymology* (1987) 136:93-103.

An additional technique is called LOCI, which stands for luminescent oxygen channeling immunoassay. The method is based on the familiar concept of bringing together two species in order to initiate a measurable event. In this instance, the two species are beads coated with antibodies. The two are brought together in pairs by an antigen. In this respect, the technique is not dissimilar to latex agglutination. However, one set of particles is labeled with a photosensitizer dye and an "antenna" molecule. This arrangement is capable of exciting molecular oxygen, which diffuses the short distance to the second bead where it initiates a chemiluminescence process by exciting a special molecule coupled to the second bead. The result is that light is emitted when the beads are joined as a consequence of an antigen-antibody reaction. However, at low analyte concentrations, there is a nonspecific binding of the beads and, consequently, a nonspecific light emission. Furthermore, the technique requires special instrumentation and, although

homogeneous, is not amenable to standard clinical analyzers. See, Ullman, E. F., et al., in *Proc. Natl. Acad. Sci. USA* (1994) 91(12):5426-30.

Yet another homogeneous assay is Microgenics' (Boehringer Mannheim) CEDIA. See, U. S. Patent Nos. 4,708,929, 5,120,653, 5,244,785, and 5,362,625. The method is based on the activity of the enzyme beta-galactosidase, which in this technique has been divided into two fragments. The acceptor fragment, EA, contains 97% of the enzyme's total mass. A smaller fragment, the donor (ED), is made by a recombinant DNA technique and contains on the order of about 80 amino acids. The ED can be engineered to contain lysine or cysteine groups at specified locations for linking.

In the CEDIA method, a hapten analog is attached to ED. Antibody binding the ED-hapten complex prevents its recombination with EA to form active enzyme. In this way, the enzyme activity is proportional to the amount of free hapten in a specimen. The sensitivity of CEDIA is perhaps one order of magnitude better than that for EMIT. For example, Microgenics has published on a vitamin B12 assay on the Cobas Mira with a sensitivity down to 100 pg/mL. See, Khanna, P.L. and Worthy, T.E., in *Diagnostics in the Year 2000*, Van Nostrand Rheinhold, Singh, P., Sharma, B.P., and Praveen, T. (Eds.) (1992) p. 2-38.

Efforts were made to apply CEDIA to high molecular weight analytes. An early effort involved binding TSH to the ED. An effect was demonstrated in this competitive format, but it was not large enough to be readily detected.

Another attempt came in the case of ferritin which has two kinds of repeating subunits. In this instance, an antibody to ferritin

is conjugated to the ED. The conjugates bunch up around ferritin in the specimen and prevent the formation of the EA-ED complex. Unlabeled antibody is added along with the conjugate to further crowd the ED and provide increased steric hindrance to complexation. This assay has been introduced in a commercial format for the Hitachi 717 analyzer with a sensitivity of 50 ng/mL. The assay requires addition of a reagent containing Ab-ED and substrate to the specimen, followed by a 5-10 minute incubation step. A second reagent containing unlabeled antibody and EA is then added. After a 3-4 minute incubation step, the absorbance is read to give the final result. The methodology is claimed to be applicable to "analytes with multivalent antigenic determinants like CRP, hepatitis surface antigen."

Recent advancements have also been made in another "homogeneous" affinity assay technology known as fluorescence polarization immunoassay (FPIA). The technology is limited by the immeasurably small signal changes that occur when the analyte mass exceeds 20,000 daltons.

Wei, A-P. and Herron, J. N., in *Anal. Chem.* (1993) 65:3372-3377, describe the use of synthetic peptides as tracer antigens in FPIA techniques reportedly to detect high molecular weight antigens. In this work, a panel of 221 octapeptides of overlapping sequence designed to span all possible eight amino acid segments present in the two chains of human chorionic gonadotropin (237 amino acid residues between the two chains) was screened with a monoclonal anti-hCG antibody. A comparison of the binding affinity of a synthetic binding peptide, GSGSRLPGPSDTC (SEQ ID NO:75), derived from the structure of

two binding peptides isolated from the panel, SRLPGPSD (SEQ ID NO:76) and RLPGPSDT (SEQ. ID NO: 77), showed that the synthetic binding peptide had a binding affinity constant (K_a) for the antibody of $1.6 \times 10^7\ M^{-1}$ versus a binding affinity constant of $4.8 \times 10^9\ M^{-1}$ observed for the naturally occurring hCG molecule. Hence, the synthetic peptide has a binding affinity that was more than two orders of magnitude lower than the naturally occurring molecule. Consequently, the synthetic peptide is ill-equipped to compete effectively with the natural hCG molecule for limited anti-hCG antibody: that is, the synthetic peptide is readily displaced by the natural hCG molecule in a competitive immunoassay format.

The Prosthetic-Group Labeled Enzyme Immunoassay (PGLEIA) is an assay in which apoglucose oxidase is inactive unless reconstituted by complexation with a ligand-labeled FAD (flavin adenine dinucleotide) analog. Antibody binding the FAD-Ligand conjugate prevents the recombination. Most of the work with this assay was done with haptens, and one study was reported in which an assay for IgG was demonstrated. See, Morris, D.L. et al., in *Anal. Chem.* (1981) 53:658-65.

In the FSIA (fluorogenic substrate-labeled immunoassay) method a ligand is covalently coupled to a fluorogenic molecule by an enzyme cleavable bond. When anti-ligand antibody is preoccupied with analyte in the specimen, the enzymatic cleavage reaction occurs and produces a fluorescent molecule. When no analyte is present, the antibody binds to the conjugate which sterically prevents the cleaving enzyme from acting. As a result, no fluorescent signal is produced. For example, the fluorogenic substrate can be a derivative of 4-methylumbelliferyl phosphate,

and the enzyme can be beta-galactosidase. Tests for IgG and IgM have been demonstrated. The IgG test has a sensitivity of 2 micrograms per ml. See, Worah, D., et al., in *Clin. Chem.* (1981) 27:673-677.

2.4 Previous Efforts of Obtaining "Mimics"

Luzzago, A., et al., in *Gene* (1993) 128:51-57, describe the selection of nonapeptides from a random nonapeptide library which bind to the monoclonal antibody H107. Two consensus sequences were described, including YXXXXXW (SEQ ID NO:78) and GSXF (SEQ ID NO:79), in which position X is variable. The value of these sequences is unclear particularly because a competition experiment set up between a biotinylated synthetic peptide containing the first consensus sequence and recombinant human H-subunit ferritin provided anomalous results. In particular, the absorption reading attributable to mAb H107 bound to biotinylated synthetic peptide actually *increases* with the addition of competing analyte. A progressive *decrease* in the absorption signal would have been expected in a well behaved system. (See, Fig. 5 of Luzzago.) Significantly, no mention of affinity assays is made.

Thus, previous efforts in the art to establish homogeneous assays have required reagent incubation times on the order of hours, have suffered from serum interferences and, in the case of the ferritin assay, required a specialized detector for measuring fluorescence. Most importantly, the existing technology allows the effective measurement of haptens, not macromolecular antigens. And where attempts have been made to measure macromolecular species, interferences and limitations persist which are not normally encountered with small hapten molecules. Also, no effective

substitutes have been discovered which faithfully reproduce the properties and characteristics of the macromolecular analytes to the point that existing techniques found effective for small molecules can be applied to the macromolecules of interest. Thus, no functional surrogates have been described, for instance, which can compete effectively with a given analyte, such as an antigen, for a limiting amount of affinity receptor, such as an antibody.

Accordingly, the present invention seeks to remedy the shortcomings in the state of the art of affinity assays, providing substances that can serve as functional surrogates of selected analytes of interest. Such substances are particularly useful for applications in the area of homogeneous immunoassays in which functional substitutes for "untractable" macromolecules are unavailable.

3. Summary of the Invention

It is therefore an object of the present invention to provide functional surrogates of analytes of interest which for all intents and purposes serve as effective substitutes for the analytes of interest, particularly when the analytes are macromolecular moieties, the detection of which have to date proved unworkable within the framework of existing affinity assay technology, such as EMIT, CEDIA, fluorescence polarization methods, and the like. It is important to stress that in the methods of the present invention, actual knowledge of the molecular structure of the segment of the analyte of interest responsible for the affinity interaction with a receptor for the analyte is neither necessary nor essential. For a given analyte of interest, all that is needed is the availability of an

affinity receptor having a selective affinity for the analyte (e.g., the availability of an antibody against an analyte of interest). Indeed, in some cases, as described further below, novel substances can be uncovered which show an affinity for an analyte of interest and which can be used as a receptor for the analyte. The formation of a complex between the novel substance and the analyte can then be detected.

A method is thus provided for determining the presence or absence of an analyte of interest in a sample by an affinity assay comprising: (a) providing (i) a labeled conjugate comprising at least one label attached to a functional surrogate capable of competing effectively with an analyte of interest for a limiting amount of an affinity receptor for the analyte, the labeled conjugate exhibiting an activity that is altered on interaction of the labeled conjugate with the affinity receptor and which activity can be measured and related to the presence or absence of the analyte in a given sample, and (ii) the affinity receptor; (b) combining the labeled conjugate and affinity receptor with a sample suspected of containing the analyte to provide a measure of the activity; (c) measuring the activity; and (d) relating the activity to the presence or absence of the analyte in the sample.

Various analytes of interest can be detected in this manner, from small haptens to large macromolecules, using available technology once the functional surrogate has been isolated and identified. Accordingly, specific embodiments of the present invention are directed to particular analytes of commercial importance, including various antigens and antibodies, and using various affinity assays well known to those of ordinary skill in the

art, including EMIT, CEDIA, and fluorescence polarization.

Another object of the invention is to provide a homogeneous immunoassay kit comprising: (a) a labeled conjugate diposed in a first container means, the labeled conjugate comprising at least one label attached to a functional surrogate of an analyte of interest, the functional surrogate capable of competing effectively with the analyte for a limiting amount of an affinity receptor for the analyte, the labeled conjugate capable of exhibiting an activity that is altered on interaction of the labeled conjugate with the affinity receptor and which activity can be measured and related to the amount of the analyte present in a given sample; and (b) disposed in a second container means the affinity receptor and, optionally, any substance required for the labeled conjugate to exhibit the activity.

Consistent with the objective of the present invention, a functional surrogate of an analyte of interest is provided which comprises a peptide having an interactive group that allows the surrogate to compete effectively with the analyte for a limiting amount of an affinity receptor for the analyte. For the practice of the above-described methods, a further object of the present invention is to provide a labeled conjugate comprising at least one label attached to a functional surrogate of an analyte of interest, the functional surrogate capable of competing effectively with the analyte for a limiting amount of an affinity receptor for the analyte, the labeled conjugate capable of exhibiting an activity that is altered on interaction of the labeled conjugate with the affinity receptor and which activity can be measured and related to the amount of the analyte present in a given sample.

The invention also provides recombinant DNA constructs

comprising a DNA sequence encoding a functional surrogate of an analyte of interest. the functional surrogate capable of competing effectively with the analyte for a limiting amount of an affinity receptor for the analyte. DNA constructs comprising a DNA sequence encoding a fusion protein of the functional surrogate of the invention are also contemplated, such as a fusion protein comprising the functional surrogate of the invention fused to the primary sequence of an enzyme label in the proximity of the label's active site. For example, the fused enzyme label can exhibit glucose-6-phosphate dehydrogenase activity.

Also provided for are transforming vector including the functional surrogate or fusion protein construct; a bacteriophage transformed by the vector encoding the functional surrogate and a microorganism transformed by the vector or infected by the bacteriophage.

Furthermore, it is an object of the present invention to provide a method of obtaining functional surrogate of an analyte of interest comprising: (a) selecting an affinity receptor exhibiting a selective affinity for an analyte of interest; (b) screening a random peptide library with the affinity receptor for a binding peptide; (c) isolating the binding peptide and identifying its primary structure. Moreover, functional surrogates of affinity receptors which exhibit a selective affinity for an analyte of interest can be obtained similarly by screening a random peptide library with the analyte.

In the method of the present invention, the identified peptide may further be prepared by known techniques, including solid phase synthesis and its capacity to compete with the analyte for a limiting amount of the affinity receptor confirmed or verified.

Alternatively, a synthetic binding peptide's capacity to selectively bind to an analyte can also be confirmed. Preferably, the binding peptide is isolated from a phage displayed random peptide library. The contents of such libraries can be designed and generated by known techniques.

The above and other objects of the invention will become readily apparent to those of ordinary skill in the relevant art from the following detailed description and drawings, in which only the preferred embodiments of the invention are described and shown, simply by way of illustration of the best mode of carrying out the invention. As is readily recognized the invention is capable of modifications within the ordinary skill of the relevant art without departing from the spirit and scope of the invention.

4. Definitions

To further assist those interested in practicing the invention, the following definitions are provided.

Activity - Any detectable, measurable phenomenon attributable directly or indirectly to the action of a particular species, such as the enzymatic activity of an enzyme, the anticoaguiant activity of heparin, the absorption spectrum of a product produced from a reaction mediated by the species in question, the emission spectrum of a fluorogenic compound, the color intensity produced in a chromogenic reaction mediated by the species in question, the current produced by an electrochemical transformation that can be related to the amount present of a species in question, the rate at which a given product is produced, photon emission, radioactivity, and the like. In the present

invention, the activity will be attributable to action of the label or the labeled conjugate, *infra*.

Affinity Receptor - A molecule that exhibits a selective affinity for an analyte of interest as defined below. Hence, an affinity receptor of a given analyte will interact or bind selectively with that analyte in the presence of other potential binding partners. An example of a common affinity receptor is an antibody against a particular antigen or one of a pair of well known affinity couples, such as biotin-avidin or protamine-heparin.

Affinity receptors are preferably antibodies, both polyclonal and monoclonal, but can be any substance, protein, nucleic acid or saccharide that binds analyte selectively, preferably specifically. Antibodies are produced by introducing an immunogen into the bloodstream of a living animal. For a review of the production of antibody reagents, see, Hurn & Chantler in "Methods in Enzymology" Vol. 70, Part A, 1980 Academic Press, eds. Van Vunakis & Langone, pp. 104-142; Kohler & Milstein *Nature* (1975) 256: 495-497.

Analyte of Interest - Any substance whose detection is of interest to the practitioner. Such substances may constitute both small and large molecules, including but not limited to haptens, immunogens, drugs of abuse, therapeutic drugs, factors, cofactors, hormones, small and large antigens, various markers, immunoglobulins, specific antibodies, proteins, glycoproteins, polysaccharides, polynucleotides, lipopolysaccharides, other lipid-containing macromolecules, and the like. Ideally, affinity receptors of the analytes of interest are available. Most preferably, analytes of interest will be any molecule for which a peptide can act as a

functional surrogate in an assay using an affinity receptor, usually an antibody. Analytes can be any compound of interest ranging from small molecule drugs and haptens (MW 100 daltons) to large proteins (MWs up to 500,000 daltons) and infectious agents such as bacteria and viruses.

Functional Surrogate - A substance that can serve as a mimic or substitute for a naturally occurring molecule, especially its functional aspects, such as the ability of that naturally occurring molecule to interact or bind selectively with an affinity receptor. Hence, a minimum requirement for a functional surrogate may be the capacity of the functional surrogate to compete with the naturally occurring molecule in question for a limited amount of affinity receptor. It is important to note that a functional surrogate may have a molecular structure (e.g., a primary sequence) that corresponds to a continuous or discontinuous epitope of a naturally occurring analyte. Alternatively, a functional surrogate may a molecular structure that differs substantially from that of the analyte or an immunoreactive group present in a segment of the analyte. Other characteristics or properties may be desirable in a given functional surrogate, including a much reduced molecular size relative to the naturally occurring molecule, a selective binding affinity (K_a) for an affinity receptor comparable to that of the naturally occurring molecule or conversely a dissociation constant (K_d) from an affinity receptor complex comparable to that of the naturally occurring molecule. The capacity of a functional surrogate to exhibit a competitive binding profile that comports to that obtained from the naturally occurring molecule may also be a desirable characteristic.

Label - Any type of marker that can be attached covalently or non-covalently to another moiety by which the presence of that moiety, such as a functional surrogate, can be detected or accounted for. The action of a label will give rise to a certain signal or activity, which can be measured. Labels can be radioisotopes, paramagnetic metals, fluorescent dyes, chemiluminescent markers, enzymes, colored or fluorescent particles (latex particles, glass beads, etc.), and the like.

Labeled Conjugate - A molecular entity that results from the molecular (e.g., protein fusion) or chemical (e.g., chemical linking) combination of a label and a functional surrogate. A labeled conjugate will have an activity associated with it, which activity becomes altered, i.e., either inhibited (decreased) or magnified (increased) on interaction of the labeled conjugate with another molecular entity, namely, an affinity receptor for a naturally occurring molecule. Like the unconjugated functional surrogate, the labeled conjugate should also be able to compete effectively with a naturally occurring molecule (typically, the analyte of interest) for a limiting amount of the affinity receptor.

Naturally Occurring- As used herein to describe a molecule, analyte or the like, "naturally occurring" can also encompass "unnatural" substances, such as those that are man-made, recombinant, non-endogeneous, non-indigenous or a pollutant, etc. The term "naturally occurring" is used merely to distinguish the analyte substance from the functional surrogate or labeled conjugate of the invention.

5. Brief Description of Drawings

FIG. 1 presents the dose-response curve obtained from increasing concentrations of peptide SEQ ID NO:58, serving as a proposed functional surrogate of hepatitis B surface antigen (HBsAg). As described in the Examples section, the increasing amount of immobilized functional surrogate of HBsAg allowed greater proportions of a fixed amount of goat anti-HBsAg to be bound to a solid support, giving rise to an increase in the "activity" or optical density measurement taken after the addition of a second antibody conjugate, rabbit anti-goat IgG horseradish peroxidase, and appropriate HRP substrate.

FIG. 2 presents the results of competitive ELISA experiments that demonstrate the substantial similarity of the competitive binding profiles exhibited by immobilized functional surrogate versus that exhibited by immobilized naturally occurring antigen. These results also support the proposition that the functional surrogates of the present invention are capable of competing effectively with the naturally occurring analyte for a limiting amount of affinity receptor (e.g., antibody).

FIG. 3 presents the Ab dilution curve results for peptide bHEP11 (SEQ ID NO:74).

FIG. 4 presents the competitive ELISA results for peptide bHEP11 (SEQ ID NO:74).

FIG. 5 presents the Ab dilution curve results for peptide bHEP2-2 (SEQ ID NO:38).

FIG. 6 presents the competitive ELISA results for peptide bHEP2-2 (SEQ ID NO:38).

FIG. 7 illustrates a scheme for the generation of a random 8

amino acid peptide library, R8C. Oligonucleotides were synthesized, converted into double-stranded DNA, cleaved with restriction enzymes, and cloned into the M13 vector, m663. The random peptide region is flanked by cysteine residues and is situated at the N-terminus of mature protein III.

FIG. 8 illustrates a scheme for the generation of another phage displayed random peptide library, R26, used in selected biopanning experiments.

FIG. 9 illustrates a scheme for the generation of the D38 phage displayed random peptide library.

FIG. 10 illustrates a scheme for the generation of the DC43 phage displayed random peptide library.

6. Detailed Description of the Invention

The invention relates to functional surrogates, most preferably binding peptides isolated from a random peptide library, useful as substitutes for a naturally occurring molecule that for one reason or another (such as undesirable size, unavailability, scarcity) cannot be used practically in a given application. Hence, having a functional surrogate in hand, certain methods can be performed, including, for example, homogenous enzyme immunoassays for large proteins. In addition, a host of other techniques previously only applicable to smaller molecules can now be carried out using the functional surrogate as a substitute for the analyte of interest. When the functional surrogate is able to compete effectively for an affinity receptor for the analyte, specific affinity binding interactions can be detected directly or indirectly, as the case may be.

By selecting a suitable affinity receptor with which to screen a random peptide library to isolate and identify binding peptides, functional surrogates of macromolecular analytes of interest can be obtained which mimic the binding properties, among other things, of the naturally occurring molecule. It is, thus, possible to design affinity assays, such as homogeneous EIAs to measure such macromolecules. After identification, the functional surrogates are synthesized using conventional techniques, including chemical synthesis, degradation of proteins, and in the case of peptides, optionally by recombinant techniques.

In a specific embodiment of the present invention, peptide epitopes (i.e., peptides corresponding to a continuous epitope found in an antigen) are isolated and characterized from a random peptide library. In other cases, peptide mimetopes (i.e., those peptides having a molecular structure that differs from that found in a continuous epitope or those peptides having a molecular structure that is a composite of the structure of a discontinuous epitope) are isolated and characterized from a random peptide library. Hence, as used herein the term "mimetope" means peptides of a defined sequence which mimic the function of epitopes of macromolecules to be measured.

In a preferred embodiment of the invention, peptides or mimetopes of a defined sequence may be used for formatting homogeneous enzyme immunoassays (EIAs) for measurement of analytes. The peptides represent specific epitopes on the analytes or mimetopes thereof which can be detected with specific affinity receptors, such as antibodies.

In general, the functional surrogates are then attached to a

label suitable for the detection method of choice. For example, a peptide mimetope is conjugated to an enzyme exhibiting glucose-6-phosphate dehydrogenase activity. The labeled peptide conjugate can then be exposed to and allowed to interact with polyclonal, monoclonal or bispecific antibodies or their fragments, such as Fab' or F(ab')$_2$, which antibodies serve as receptors for the naturally occurring analyte. The complex formed from the interaction, typically a binding interaction, of labeled peptide with antibody results in the inhibition of enzyme activity. If the activity is monitored, then the observed activity can be related to the amount of analyte present in a given sample, especially when the observed activity is compared to that observed from at least one control (i.e., a sample with a known amount of analyte, such as below or above the detection limit to provide a negative or a positive control). Of course, either specific antibody against the analyte of interest or competing analyte in the sample can be detected in this manner. The reaction between labeled peptide conjugate and antibody, or fragment, is relatively selective, preferably specific, and takes place preferably, but not necessarily, at the antigen binding site on the antibody.

Peptides of 5-35, preferably less than 15, amino acid residues or more in length that include core sequences representing single epitopes, epitope composites or mimetopes thereof found in large molecules can be chemically synthesized. The desired peptide sequence is deduced from the nucleotide sequence of DNA inserts found in isolated phage clones from phage displayed random peptide libraries which bind to the target affinity receptor following the selection procedure, e.g., "biopanning" experiments. The target

affinity receptors are preferably polyclonal antibodies, most preferably specific monoclonal antibodies. In specific embodiments of the invention, individual functional surrogate peptides may contain from about 4 to about 100 amino acid residues. Still other peptides may have about 35 amino acids or less, such as 6-25 amino acids. The number of residues is somewhat variable because of the possible conformational requirements of the functional binding region of the surrogate and the need in some cases to have additional flanking sequences. Hence, preferred functional surrogates may have 8-14 amino acid residues, while others may have 8-20 amino acid residues (see, Tables 1 and 2). To reiterate, the use of the random peptide library means that the possible molecular structures of potential binding peptides are not limited to or dictated solely by the primary sequence of a proteinaceous analyte. Consequently, binding peptides can potentially be isolated corresponding to known epitopes, to previously unknown epitopes or to wholly unrelated but functionally equivalent structures of immunogenic analyte segments.

TABLE 1. SYNTHETIC FERRITIN PEPTIDES

(Note: all peptides bear free alpha amine and omega carboxyl, unless otherwise annotated.)

SEQ. ID. NO.	SEQUENCE
1	SGGRALFQS
2	*SGGRALFQS
3	SGGRALFQS*
4	*-eca-RGGRALFQS
5	Ac-D-βala-βala-SGGRALFQS
6	Ac-SGGRALFQS-βala-βala-D
7	SGGRALFQS-βala-βala-D
8	Ac-D-βala-Y-βala-SGGRALFQS
9	Ac-SGGRALFQSD-βala-Y-βala
10	Ac-SGGRALFQS-eCA-COOH
11	Ac-RGGRALFQS-eCA-Y-eCA-D
12	RGGRALFQSBBYBC
13	SSINPTPSD
14	*SSINPTPSD
15	LRQPAVSGGR SLFQNLDPSR
16	LRQPAVSGGR SLFQNLDSR
17	RGGRALFQS-eca-KK
18	KK-eca-RGGRALFQS
19	*ESSALFQ
20	*E-βala-SALFQS
21	Ac-E-βala-SALFQS
22	SSLFQE
23	*SSLFQE
24	RAFFRD
25	*RAFFRD

26	KYGGMSLFQSQMTAGHHAGT
27	TAKEGSVGGASLFLELRAQC
28	ESSLFQ
29	ECSSLFQC
30	EGGASLF
31	ECGGASLFC

TABLE 2. SYNTHETIC HEPATITIS PEPTIDES

(Note. all peptides bear free alpha amine and omega carboxyl groups, unless otherwise annotated. Also, all peptides with two cysteines are cylized as cystine.)

SEQ. ID. NO.	SEQUENCE
32	*CTGPRHLC
33	*SDHPLYSR
34	*LPGPPHLS*
35	LPGPPHLS
36	Ac-LPGPPHLS
37	C-oK-LPGPPHLS C-oK-LPGPPHLS
38	*C-oK-LPGPPHLS *C-oK-LPGPPHLS
39	Ac--DC-eCA-LPGPPHLS Ac--DC-eCA-LPGPPHLS
40	Ac--LPGPPHLS{E
41	Ac--LPGPPHLS-Ok-{{-Ok
42	*STTSIGPTK
43	RCPSDGNCY

44	*RCPSDGNCY
45	*PSDGN
46	*RSPSDGNSY
47	*CPSDGNC
48	*SPSDGN
49	*CEEGAVLPKC
50	*-eCA-CEEGAVLPKC
51	CTKPSDGNYC
52	*CTKPSDGNYC
53	Ac-oK-CTKPSDGNYC
54	CTKPSSGNYC
55	RCTKPSDGNYC
56	*RCTKPSDGNYC
57	eca-CTKPSDGNYC
58	*eca-CTKPSDGNYC
59	CO-CH_2——— S —— NH-eCA-KTRPSDGNYC-$CONH_2$ NH_2
60	CO-CH_2——— S —— NH-eCA-KTRPSDGNYC-$CONH_2$ biotin-NH_2
61	KCTKPSDGNCK

SUBSTITUTE SHEET (RULE 26)

62	*KCTKPSDGNCK
63	*KCTKPSDGNCKK
64	ECTKPSDGNCE
65	*ECTKPSDGNCE
66	@ECTKPSDGNCE
67	CTKPSDGNCK
68	*CTKPSDGNCK
69	*@CTRPSDGNYC
70	*CKPSDGNC
71	*CTKPSDGNC
72	*CPSDGNYC
73	*CKPSDGNYC
74	*@CTKPSDGNYC@Y

For Tables 1 and 2, the "*" symbol refers to a biotin label; "@" or "eca" stands for epsilon amino caproic acid; "OK" refers to a "sideways" attached lysine, with the N-terminal peptide bond forming at the epsilon amino group; a "{" symbol refers to a branching lysine (MAP-"multiple antigenic peptide" technology discussed further below); "Ac" is an acetyl group; and intramolecular bonds, typically cystine groups, are indicated by the solid lines.

Accordingly, for example, SEQ. ID. No. 41 represents a tetrameric antigenic peptide, in which four copies of the peptide Ac-LPGPPHLS-OK (SEQ ID NO:369) are attached to the four animo groups of the poly-lysine core of a four-branch MAP represented by the symbol "{{" (MAP4). The structure of MAP4 attached at the C-terminal end to a "sideways" lysine (OK) is

$$\begin{array}{r} H_2NCH_2CH_2CH_2CH_2CH(NH_2)CONH \\ | \\ H_2NCH_2CH_2CH_2CH_2CH(NH_2)CONHCH_2CH_2CH_2CH_2CHCO\text{-}OK. \end{array}$$

The use of MAP technology to make high-density synthetic peptide systems has been described. See, for example, Tam, J.P., in *Proc. Natl. Acad. Sci. USA* (1988) 85:5409-5413; Tam, J.P. and Zavala, F. in *J. Immunolog. Meth.* (1989) 124:53-61; Briand, J.-P., et al., in *J. Immunolog. Meth.* (1992) 156:255-265; MAP technology has also been extended to solid phase synthesis on resins. See, for example, Applied Biosystems' *User Bulletin* (1992) No. 34.

Such small functional surrogates can be easily conjugated to labels, such as G6PDH by conventional techniques presently in use to couple haptens and drugs to comparable labels. Such coupling can be accomplished without appreciable loss of activity attributable to the label, and, hence, the same activity can be attributed to the labeled conjugate.

The use of these peptide epitope/mimetope conjugates allows the construction of a variety of affinity assays, as mentioned earlier, including homogeneous EMIT-type, CEDIA, and "TDX" (fluorescence polarization) assays for the measurement of large polypeptides and proteins analytes.

Examples of analytes which may be detected by the method of the invention include, but are not limited to ferritin, follicle stimulating hormone (FSH), thyroid-stimulating hormone (TSH), human growth hormone (hGH), immunoglobulin E (IgE), prolactin, parathyroid hormone (PTH), and human placental lactogen (HPL). In the area of fertility/pregnancy, human chorionic gonadotropin (hCG) and human luteinizing hormone (hLH) can be detected in addition to FSH. Infectious agents may also be assayed, including cytomegalovirus (CMV), chlamydia, streptomycin A, rubella, toxoplasma, herpes, and hepatitis. Also, the presence or absence of cardia markers, such as CK-MB, myoglobin, myosin light chain, and troponin, in addition to tumor markers, such as carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), PSA, and CA125 can be determined. The method of the invention also avails itself to rapid allergy screening.

The analytes of interest to this invention are broad and varied. They may be characterized by being monoepitopic or polyepitopic. The polyepitopic analytes will normally be poly (amino acids), i.e., polypeptides and proteins, polysaccharides, nucleic acids, and combinations thereof. Such combinations or assemblages include bacteria, viruses, chromosomes, genes, mitochondria, nuclei, cell membranes, and the like.

For the most part, the polyepitopic analytes employed in the subject invention will have a molecular weight of at least about 5,000 more usually at least about 10,000. In the poly(amino acid) category, the poly(amino acids) of interest will generally be from about 5,000 to 5,000,000 molecular weight, more usually from about 20,000 to 1,000,000 molecular weight; among the hormones

of interest, the molecular weights will usually range from about 5,000 to 60,000 molecular weight.

The following are classes of proteins that are related by structure and are potential analytes of interest: protamines, histones, albumins, globulins, scleroproteins, phosphoproteins, mucoproteins, chromoproteins, kipoproteins, nucleoproteins, glycoproteins, unclassified proteins, e.g. somatotropin, prolactin, insulin, pepsin.

In addition, a number of proteins found in the human plasma are important clinically and include: Prealbumin, Albumin, α_1-Lipoprotein, α_1-Acid glycoprotein, α_1-Antitrypsin, α_1-Glycoprotein, Transcortin, Postalbumin, α_1-glycoprotein, $\alpha_{1\chi}$-Glycoprotein, Thyroxin-binding globulin, Inter-α-trypsin-inhibitor, Gc-globulin, Haptoglobin, Ceruloplasmin, Cholinesterase, α_2-Lipoprotein(s), α_2-Macroglobulin, α_2-HS-glycoprotein, Zn-α_2-glycoprotein, α_2-Neuramino-glycoprotein, Erythropoietin, ß-lipoprotein, Transferrin, Hemopexin, Fibrinogen, Plasmimogen, $ß_2$-glycoprotein I, $ß_2$-glycoprotein II, Immunoglobulin G, (IgG) or $_\gamma$G-globulin, Mol. formula: $\gamma_2\kappa_2$ or $\gamma_2\lambda_2$, Immunoglobulin A (IgA), or $_\gamma$A-globulin, Mol. formula: $(\alpha_2\kappa_2)^n$ or $\alpha_2\lambda_2)^n$, Immunoglobulin M, (IgM) or $_\gamma$M-globulin, Mol. formula: $\mu_2\kappa_2)^5$ or $(\mu_2\lambda_2)^5$, Immunoglobulin D (IgD), or $_\gamma$D-Globulin ($_\gamma$D), Mol. formula: $(\delta_2\kappa_2)$ or $(\delta_2\lambda_2)$, Immunoglobulin E (IgE), or $_\gamma$E-Globulin ($_\gamma$E), Mol. formula: $(\varepsilon_2\kappa_2)$ or $(\varepsilon_2\lambda_2)$, Free κ and γ light chains, Complement factors: C'1 (C'1q, C'1r, C'1s), C'2, C'3 ($ß_1$A, α_2D), C'4, C'5, C'6, C'7, C'8, C'9. Potential analytes of interest, such as:

BLOOD CLOTTING FACTORS	
International designation	Name

I	Fibrinogen
II	Prothrombia
IIa	Thrombin
III	Tissue thromboplastin
V and VI	Proaccelerin, accelerator globulin
VII	Proconvertin
VIII	Antihemophilic globulin (AHG)
IX	Christmas factor, plasma thromboplastin component (PTC)
X	Stuart-Prower factor, autoprothrombin III
XI	Plasma thromboplastin antecedent (PTA)
XII	Hagemann factor
XIII	Fibrin-stabilizing factor

Important protein hormones include: Peptide and Protein Hormones such as Parathyroid hormone, (parahormone), Thyrocalcitonin, Insulin, Glucagon, Relaxin, Erythropoietin, Melanotropin, (melanocyte-stimulating hormone; intermedin), Somatotropin, (growth hormone), Corticotropin, (adrenocorticotropic hormone), Thyrotropin, Follicle-stimulating hormone, Luteinizing hormone, (interstitial cell-stimulating hormone), Leuteomammotropic hormone, (luteotropin, prolactin), Gonadotropin, (chorionic gonadotropin);

Tissue Hormones, Secretin, Gastrin, Angiotensin I and II, Bradykinin, Human placental lactogen; and Peptide Hormones from the Neurohypophysis, such as Oxytocin, Vasopressin, Releasing factors (RF), CRF, LRF, TRF, Somatotropin-RF, GRF, FSH-RF,

PIF, MIF.

Other macromolecular analytes of interest are mucopolysaccharides and polysaccharides.

Illustrative antigenic polysaccharides derived from microorganisms are as follows:

Species of Microorganisms	Hemosensitin Found in
Streptococcus pyogenes	Polysaccharide
Diplococcus pneumoniae	Polysaccharide
Neisseria meningitidis	Polysaccharide
Neisseria gonorrhoeae	Polysaccharide
Corynebacterium diphtheriae	Polysaccharide
Actinobacillus mallei; Actinobacillus whitemori	Crude extract
Francisella tularensis	Lipopolysaccharide Polysaccharide
Pasteurella pestis	
Pasteurella pestis	Polysaccharide
Pasteurella multocida	Capsular antigen
Brucella abortus	Crude extract
Heamophilus influenzae	Polysaccharide
Haemophilus pertussis	Crude
Treponema reiteri	Polysaccharide
Veillonella	Lipopolysaccharide
Erysipelothrix	Polysaccharide
Listeria monocytogenes	Polysaccharide
Chromobacterium	Lipopolysaccharide

Mycobacterium tuberculosis	Saline extract of 90% phenol extracted mycobacteria and polysaccharide fraction of cells and tuberculin
Klebsiella aerogenes	Polysaccharide
Klebsiella cloacae	Polysaccharide
Salmonella typhosa	Lipopolysaccharide Polysaccharide
Salmonella typhi-murium; Salmonella derby	Polysaccharide
Salmonella pullorum	
Shigella dysenteriae	Polysaccharide
Shigella flexneri	
Shigell sonnei	Crude. polysaccharide
Rickettsiae	Crude extract
Candida albicans	Polysaccharide
Entamoeba histolytica	Crude extract

The microorganisms which are assayed may be intact, lysed, ground or otherwise fragmented, and the resulting composition or portion, e.g., by extraction, assayed. Microorganisms of interest include: Corynebacteria (*Cornebacterium diptheriae*), Pneumococci (*Diplococcus pneumoniae*), Streptococci (*Streptococcus pyogenes, Streptococcus salivarus*), Staphylococci (*Staphylococcus aureus, Staphylococcus albus*) Neisseriae (*Neisseria meningitidis, Neisseria gonorrheae*) Enterobacteriaciae, (*Escherichia coli. Aerobacter aerogenes, Klebsiella pneumoniae, Salmonella typhosa, Salmonella chloeracsuis, Salmonella typhimurium, Shigella dysenteriae, Shigella schmitzii, Shigella arabinotarda, Shigella flexneri, Shigella*

boydii, Shigella Sonnei);

Other enteric bacilli. (*Proteus vulgaris, Proteus mirabillis, Proteus morgani, Pseudomonas aeruginosa, Alcaligenes faecalis, Vibrio cholerae)*; Hemophilus-Bordetella group (*Hemophilus influenzae, H. ducreyi, H. hemophilus, H. aegypticus, H. paraiufluenzae, Bordetella pertussis*), Pasteurellae (*Pasteurella pestis, Pasteurella tulareusis)*, Brucellae (*Brucella melitensis Brucella abortus, Brucella suis)*, Aerobic Spore-forming Bacilli *(Bacillus anthracis, Bacillus subtilis, Bacillus megaterium, Bacillus cereus*), Anaerobic Spore-forming Bacilli (*Clostridium botulinum, Clostridium tetani Clostridium perfringens, Clostridium novyi, Clostridium septicum, Clostridium histolyticum, Clostridium tertium, Clostridium bifermentans, Clostridum sporogenes*);

Mycobacteria (*Mycobacterium tuberculosis hominis, Mycobacterium bovis, Mycobacterium avium, Mycobacterium leprae, Mycobacterium paratuberculosis*), Actinomycetes (fungus-like bacteria) (*Actinomyces israelii, Actinomyces bovis, Actinomyces naeslundii, Nocardia asteroides, Nocardia brasilinesis*);

The Spirochetes (*Treponema pallidum, Spirillum minus, Treponema pertenue, Streptobacillus moniliformis, Treponema carateum, Borrelia recurrentis, Leptospira icterohemorrhagiae, Letospira canicola*), Mycoplasmas (*Mycoplasma pneumoniae*, Other pathogens (*Listeria monocytogenes, Erysipelothrix rhusiopathiae, Streptobacillus moniliformis, Donvania granulomatis, Bartonella bacilliformis*), Rickettsiae (bacteria-like parasites) (*Rickettsia prowazekii, Rickettsia mooseri, Rickettsia rickettsii, Rickettsia conori, Rickettsia australis, Rickettsia sibiricus, Rickettsia akari, Rickettsia tsutsugamushi, Rickettsia burnetii, Rickettsia quintana*);

Chlamydia (Chlamydia agents), Fungi, *Cryptococcus neoformans, Blastomyces dermatidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Candida albicans, Aspergilus fumigatus, Mucor corymbifer (Absidia corymbifera), Rhizopus oryzae, Rhizopus arrhizus, Rhizopus nigricans, Sporotrichum schenkii, Fonsecaea pedrosoi, Fonsecaea compacta, Fonsecaea dermatitidis, Cladosporium carrionii, Phialophora verrucosa, Aspergillus nidulans*, Madurella mycetomi, (*Madurella grisea, Allescheria boydii, Phialosphora jeanselmei, Microsporum gypseum, Trichophyton mentagrophytes, Keratinomyces ajelloi, Microsporum canis, Trichophyton rubrum, Microsporum andouini*);

Viruses, Adenoviruses, Herpes viruses, (*Herpes simplex*, Varicella (Chicken pox), Herpes Zoster (Shingles), Virus B, Cytomegalovirus), Pox Viruses, (Variola (smallpox), Vaccinia, *Poxvirus bovis*, Paravaccinia, *Molluscum contagiosum*), Picronaviruses (Poliovirus, Coxsackievirus, Echoviruses, Rhinoviruses);

Myxoviruses (Influenza (A, B and C), Parainfluenza (1-4), Mumps Virus, Newcastle Disease Virus, Measles Virus, Rinderpest Virus, Canine Distemper Virus, Respiratory Syncytial Virus, Rubella Virus), Arboviruses (Eastern Equine Eucephalitis Virus, Western Equine Eucephalitis Virus, Sindbis Virus, Chikungunya Virus, Semliki Forest Virus, Mayora Virus, St. Louis Encephalitis Virus, California Encephalitis Virus, Colorado Tick Fever Virus, Yellow Fever Virus, Dengue Virus), Reoviruses (Reovirus Types 1-3), Hepatitis (Hepatitis A Virus, Hepatitis B Virus, Hepatitus C Virus), Tumor Viruses (Rauscher Leukemia Virus, Gross Virus,

Maloney Leukemia Virus).

The monoepitopic ligand analytes will generally be from about 100 to 2,000 molecular weight, more usually from 125 to 1,000 molecular weight. The analytes of interest include drugs, metabolites, pesticides, pollutants, and the like. Included among drugs of interest are the alkaloids. Among the alkaloids are morphine alkaloids, which includes morphine, codeine, heroin, dextromethorphan, their derivatives and metabolites; cocaine alkaloids, which includes cocaine and benzoyl ecgonine, their derivatives and metabolites; ergot alkaloids, which includes the diethylamide of lysergic acid; steroid alkaloids; iminazole alkaloids; quinazoline alkaloids; isoquinoline alkaloids; quinoline alkaloids; which includes quinine and quinidine; diterpene alkaloids, their derivatives and metabolites.

The next group of drugs includes steroids, which includes estrogens, gestrogens, androgens, adrenocortical, bile acids, cardiotonic glycosides and aglycones, which includes digoxin and digoxigenin, saponins and sapogenins, their derivatives and metabolites. Also included are the steroid mimetic substances, such a diethyl stilbestrol.

The next group of drugs comprise cyclic lactams having from 5 to 6 membered rings, which include the barbiturates, diphenyl hydantoin, and their metabolites.

The next group of drugs is aminoaklyl benzenes, with alkyl of from 2 to 3 carbon atoms, which includes the amphetamines, catecholamines, which includes ephedrine, L-dopa, epinephrine, narceine, papaverine, their metabolites and derivates.

The next group of drugs is benzheterocyclics which include

oxazepam, chlorpromazine, tegretol, imipramine, their derivatives and metabolites, the heterocyclic rings being azepines, diazepines and phenothiazines.

The next group of drugs is purines, which includes theophylline, caffeine, their metabolites and derivatives.

The next group of drugs includes those derived from marijuana, which includes cannabinol and tetrahydrocannabinol.

The next group of drugs includes the vitamins such as A, B, C, D, E and K.

The next group of drugs is prostaglandins, which differ by the degree and sites of hydroxylation and unsaturation.

The next group of drugs is antibiotics, which include penicillin, chloromycetin, actinomycetin, tetracycline, terramycin, their metabolites and derivatives.

The next group of drugs is the nucleosides and nucleotides, which include ATP, NAD, FMN, adenosine, guanosine, thymidine, and cytidine with their appropriate sugar and phosphate substituents.

The next group of drugs is miscellaneous individual drugs which include methadone, meprobamate, serotonin, meperidien, amitriptyline, nortriptyline, lidocaine, procaineamide, acetylprocaineamide, propanolol, griseofulvin, butryophenones, antihistamines, anticholinergic drugs, such as atropine, their metabolites and derivatives.

The next group of compounds is amino acids and small peptides which include thyroxin, triiodothyronine, oxytocin, ACTH, angiotensin, gentamycin, met- and leu-enkephalin their metabolites and derivatives.

Metabolites related to diseased states include spermine, galactose, phenylpyruvic acid, and porphyrin type 1.

Among pesticides of interest are polyhalogenated biphenyls, phosphate esters, thiophosphates, carbamates, polyhalogenated sufenamides, their metabolites and derivatives.

For receptor analytes, the molecular weights will generally range from 10,000 to 2 x 10^6, more usually from 10,000 to 10^6. For immunoglobulins IgA, IgG, IgE and IgM, the molecular weights will generally vary from about 160,000 to about 10^6.

Enzyme analytes will normally range from about 10,000 to 600,000 in molecular weight. Natural receptors vary widely, generally being at least about 25,000 molecular weight and may be 10^6 or higher molecular weight including such materials as avidin, thyroxine binding globulin, thyroxine binding prealbumin, transcortin, etc.

In addition, numerous hybridomas have been deposited and are available from the ATCC. Such hybridomas produce antibodies that can serve as affinity receptors for use in biopanning experiments to identify functional surrogates of specific antigens. See, for example, ATCC catalog of Cell Lines & Hybridomas, 7th Ed. (1992). See, for example, pp. 319-332 (secreted mAb).

Specific peptides of a defined sequence can be produced *in vitro* by synthesis or by chemical or enzymatic cleavage. Alternatively, such peptide can be produced *in vivo* by a natural process. The peptides preferably have a MW of about 2000 or less, and must be capable of competing effectively in the presence of naturally occurring analyte for a limited amount of affinity receptor (e.g., antibody). The peptide should preferably exert only a

minimal effect, if any, on the activity of a label when conjugated to that label. As stated elsewhere, labels can comprise fluorescent markers, enzymes, enzyme substrates, and the like. Examples of fluorescent markers include fluoresceins, rhodamines, cyanins, eosins, and the like. A preferred enzyme is glucose-6-phosphate dehydrogenase. Other suitable candidate enzymes are lysozyme or beta-galactosidase. A suitable candidate enzyme is one whose activity is little affected by conjugation to the functional surrogate, but is greatly affected by binding of affinity receptor to the labeled conjugate.

Table 3 lists some additional enzymes that may be suitable for use in the present invention, along with their enzyme substrates.

TABLE 3. LIST OF ENZYMES AND THEIR SUBSTRATES

I. Hydrolases Carbohydrases	
Amylase. Lactase. Maltase. Sucrase. Emulsion	Carbohydrates. Starch. dextrin. etc. Lactose. Maltose. Sucrose ß-Glucosides and derivatives
II. Nucleases	
Polynucleotidase, Nucleotidase	Nucleic acid, Nucleotides
III. Arginase	
Arginase. Urease. Glutaminase. Transaminase	Amino compounds and amides. Arginine, Utea, Glutamine. Glutamic acid, etc.
IV. Purine Deaminases	
Adenase. Guanase	Adenine, Guanine
V. Peptidases	
Aminopoluypeptidase, Carboxypeptidase. Dipeptidase. Prolinase	Polypeptides. Dipeptides. Proline peptides
VI. Proteinases	
Pepsin. Trypsin. Cathepsin, Rennin. Chymotrypsin. Papain, Ficin	Proteins. proteoses. Casein proteins, peptones
VII. Esterases	
Lipase, Esterases. Phosphatases. Sulfatases. Cholinesterase	Fats. ethyl buryraate. etc., esters of phosphoric acid. esters of sulfuric acid. Acetylcholine

VIII. Iron Enzymes	
Catalase. Cytochrome oxidase. Peroxidase	Hydrogen peroxide. reduced cytochrome C in the presence of oxygen. a large number of phenols. aromatic amines. etc.. in the presence of H_2O_2
IX. Copper Enzymes	
Tyrosinase (poly-phenol-oxidase, mono-phenoloxidase, Absorbic acid oxidase	Various phenolic compounds. Ascorbic acid in the presence of oxygen
X. Enzymes Containing Co-Enzymes I and/or II	
Alcohol dehydrogenase. Malic dehydrogenase. Isocriterie dehydrogenase. Lactic dehydrogenase. ß-Hydroxybutyric dehydrogenase, Glucose dehydrogenase, Glycerophosphate dehydrogenase. Aldehyde dehydrogenase	Ethyl alcohol and other alcohols. L( ) Malic acid. L-Isocritic acid. Lactic acid. L-ß-Hydroxybutyric acid, D-Glucose. Robinson ester (hexose-6-phosphate) Glycerophosphate. Aldehydes
XI. Enzymes Which Reduce Cytochrome	
Succinic dehydrogenase (as ordinarily prepared)	Succinic acid
XII. Yellow Enzymes	
Warburg's old yellow enzyme Diaphorase. Haas enzyme. Xanthine oxidase. D-amino acid oxidase. L-Amino acid oxidases. TPN-Cytochrome C reductase. DPN-Cytochrome reductase	Reduced co-enzyme II. Hypoxanthine xanthine. aldehydes. reduced co-enzyme I, etc., D-amino acids+ O_2 L amino acids. reduced co-enzymes II and cytochrome C. reduced co-enzymes II and cytochrome C

XIII. <u>Hydrases</u>	
Fumarase, Aconitase Enolase	Fumaric acid + H_2O Citric acid, 2-Phosphoglyceric acid
XIV. <u>Mutases</u>	
Glyoxalase substituted glyoxals	Methyl glyoxal and other
XV. <u>Desmolases</u>	
Zymohexase (aldolase), Carboxylase, ß-Keto carboxylases, Amino acid decarboxylases, Carbonic anhydrase	Fructose 1,6-diphosphate, pyruvic acid, ß-Keto acids L-Amino acids, Carbonic acid
XVI. <u>Other Enzymes</u>	
Phosphorylase, Phosphohexdo-isomerase, Hexokinase Phosphoglucomutase	Starch or glycogen and phosphate, Glycose-6-phosphate, Adenosine-triphosphate, Glucose-1-phosphate

As mentioned previously, specific binding peptides having a defined sequence can be obtained in a variety of ways, including isolation, subsequent to specific or non-specific enzyme digestion or chemical degradation of macromolecule; in vivo or in vitro production by transformed cells, tissue culture, and transgenic animals.

The specific binding peptide, such as any one of those listed in Tables 1 and 2, can then be conjugated by conventional methods to a label, preferably an enzyme. Conjugation methods such as those disclosed in U.S. Patent Nos. 4,423,143 and 4,560,648 can be

used, substituting the desired peptide, with or without the use of additional linker groups, for the high molecular weight proteins discussed in the examples provided in these patents, whose complete disclosure is incorporated herein by reference. In the alternative, the specific binding peptide can be produced by recombinant techniques as a fusion protein comprising the specific binding peptide and a second polypeptide, preferably an enzyme label. Most preferably, a functional surrogate of the present invention is incorporated into the primary structure of an enzyme, such as G6PDH or ß-galactosidase, in the proximity of its active site. Interaction of the "fusion" enzyme with affinity receptor for the functional surrogate would thus lead to inhibition of the enzyme activity. Hence, DNA constructs comprising DNA sequences encoding an enzyme of choice can be modified by conventional methods to include a DNA insert encoding a functional surrogate. Expressed fusion enzyme can be selected for the desired activity. The inhibition of this activity on exposure of the fusion enzyme to the appropriate affinity receptor is then observed as in the chemically linked combinations of enzyme and functional surrogate. See, e.g., U.S. Patent No. 5,362,625 for representative preparative recombinant techniques involving modified enzymes.

And while the functional surrogates can be used in a wide variety of affinity assays, homogeneous immunoassays would particularly benefit because of the existing inability of such assays to provide accurate, sensitive information regarding macromolecular analytes.

In a preferred embodiment of the invention, a homogeneous fluorescence polarization immunoassay method is provided.

Fluorescence polarization immunoassay is made possible by the property of fluorescence polarization being affected by the molecular environment. The analyte to be measured (usually a drug, hapten or large molecule in the form of a surrogate) is chemically conjugated to a fluorescent label. A competition is set up between unlabeled analyte in the sample (drug, hapten or large molecule) and the labeled conjugate for limited antibody. The reaction is followed in a Polarization Fluorometer. Using a functional surrogate of the large molecule (or of the drug or hapten for that matter), the fluorescence polarization technique can now be applied as easily and conveniently to the detection of macromolecular analytes. Subsequent combination with an affinity receptor (antibody) alters the molecular environment of the fluorophore due to the presence of a large antibody molecule. Thus, the polarizing property of the fluorophore is altered and monitoring of the reaction and quantitation of analyte can be achieved by following the fluorescence polarization.

More preferably a fluorescent labeled peptide serving as a functional surrogate of a naturally occurring analyte is used and which would compete for limited antibody with the epitope on a macromolecular analyte (supplied as sample).

In a CEDIA format, two fragments (ED and EA) of the enzyme beta-galactosidase are produced by recombinant techniques. Neither of the fragments alone has enzyme activity. When mixed together the 2 fragments combine to form active enzyme. Analyte to be measured is chemically conjugated to the ED fragment, and this conjugated ED fragment can still combine with EA fragment to form active enzyme. However, if ED conjugate is bound by

antibody to analyte then it is unable to combine with EA to form active beta-galactosidase and produce a signal. In this context, "fusion" enzyme fragments comprising ED fragment fused to functional surrogate can also be contemplated.

Therefore, if a competition is set up between analyte (provided in a sample) and ED conjugate for limited antibody, the presence of analyte in the sample would leave ED available to combine with EA and give active enzyme. Hence, monitoring for beta-galactosidase activity gives a measure of analyte in the sample.

As already illustrated in the case of EMIT and Fluorescence Polarization, use of small functional surrogate (e.g. a peptide) to conjugate to the ED fragment that would compete for antibody with the epitope on a larger molecule (supplied as sample), would allow for the measurement of larger analytes by CEDIA.

Accordingly, a method of determining the presence or absence of an analyte of interest in a sample by an affinity assay in accordance with the present invention includes the steps of: (a) providing (i) a labeled conjugate comprising at least one label attached to a functional surrogate capable of competing effectively with an analyte of interest for a limiting amount of an affinity receptor for the analyte, the labeled conjugate exhibiting an activity that is altered on interaction of the labeled conjugate with the affinity receptor and which activity can be measured and related to the presence or absence of the analyte in a given sample, and (ii) the affinity receptor; (b) combining the labeled conjugate and affinity receptor with a sample suspected of containing the analyte to provide a measure of the activity; (c) measuring the activity; and (d) relating the activity to the presence or absence of the analyte in

the sample. In specific embodiments of the invention this interaction is a binding interaction. Moreover, the functional surrogate may be further characterized as exhibiting a competitive binding profile that is substantially similar to that exhibited by the analyte for the affinity receptor. (See, for example, FIGS. 2, 4, and 6.) In addition, the functional surrogate is further characterized as exhibiting a selective binding affinity (K_a) for the affinity receptor which is substantially similar to that exhibited by the analyte. Hence, while some past work may have used certain octapeptides as a substitute for human chorionic gonadotropin, the binding affinity of the labeled peptide was some two orders of magnitude less than the binding affinity of the natural analyte for anti-hCG.

In a particular method of the invention, step (d), the relating step, comprises comparing the activity with that obtained from at least one control to determine the presence or absence of the analyte in the sample. Preferably, two controls are used, one for a negative result and the second for a positive reading.

As mentioned above, it is preferred that the functional surrogate is obtained by screening a random peptide library with one or more affinity receptors of the analyte.
Most preferably, the random peptide library comprises a plurality of peptides whose structures are not dictated by the primary sequence of the analyte. In specific embodiments, the molecular structure of the functional surrogate may nonetheless correspond to an epitope of the analyte. However, the structure of the epitope may have been previously unknown and would not have been discovered but for the present methods.

And in other cases, the molecular structure of the functional

surrogate differs from that of a known epitope of the analyte. In this case, the structure of the surrogate may be a composite of a discontinuous epitope or may simply have little or no correlation with the naturally occurring sequence structure. Hence, in certain cases, the molecular structure of the functional surrogate does not include a primary sequence of eight or more continuous amino acid residues which can be found along the naturally occurring sequence of the analyte.

While the functional surrogate of the invention may be of any size suitable for the affinity assay of choice, it preferably has a molecular weight of about 2000 daltons or less. Most preferably, the functional surrogate comprises a peptide of about 1500 daltons or less.

In accordance with the invention, the prescribed combining step may be carried out such that it includes the formation of an affinity receptor-labeled conjugate complex. The combining step may further comprise displacing the labeled conjugate from the complex with the analyte (i.e., a sequential displacement step). Still in other embodiments, the combining step comprises providing competition among the analyte and the labeled conjugate for the affinity receptor. Moreover, the combining step may comprise forming an affinity receptor-analyte complex. Subsequently, the combining step further comprises forming an affinity receptor-labeled conjugate complex. In yet a more specific embodiment of the invention, the combining step comprises (i) mixing the affinity receptor and sample, and (ii) adding the labeled conjugate to the resulting mixture.

Samples suspected of containing an analyte of interest may

be from a wide variety of sources. For example, the sample may be a biological fluid, including but not limited to urine, semen, saliva, sweat, blood, serum, plasma, cerebrospinal fluid, tears, vaginal or nasal fluids. In addition, the sample may be obtained from a cell-free extract.

In selected embodiments of the invention, the label is selected from the group consisting of a chromogenic gent, a UV absorber, a fluorescent molecule, a chemiluminescent compound, an enzyme, an enzyme fragment, an enzyme substrate, or a group having the potential for exhibiting at least one of the above-recited activities (e.g., after cleavage of a bond). Preferably, the label comprises an enzyme, most preferably one that exhibits glucose-6-phosphate dehydrogenase (G6PDH) activity so that the assay can be performed on a standard clinical chemistry analyzer. If the enzyme has G6PDH activity, then a suitable substrate for the enzyme would include glucose-6-phosphate (G6P) and nicotinamide adenine dinucleotide (NAD). Other suitable enzymes may be those that exhibit lysozyme activity or beta-galactosidase activity.

Various methods of measuring the activity of the labeled conjugate are available depending on the nature of the label. For example, the activity can be measured as a function of the change in the intensity of an absorbance or an emission spectrum, as a function of the change in the polarization or anisotropy of a fluorescence spectrum, as a function of the change in the number of particles observed in a sample mixture, as a function of the change in the amount of a product that is produced by a transformation mediated by the label, as a function of time or a rate of change, to name a few.

As mentioned above, the analyte can be any molecule of interest, such as a polysaccharide, a polynucleotide, a glycoprotein or a lipid-containing macromolecule. In preferred embodiments, the analyte is a fertility/pregnancy-related hormone, is related to an infectious disease (e.g., a bacterium or a virus), is a cardiac marker or a tumor marker. In still other embodiments, the preferred analytes have already available affinity receptors having a selective, most preferably specific, binding affinity for the analyte, including certain allergens. The molecular weight of the analyte may vary along a wide range, e.g., 200 to 500,000 daltons. Preferably, the analyte has a molecular weight in the range of about 1,000 to about 500,000 daltons, more preferably in the range of about 10,000 to about 200,000 daltons. However, all analytes having a molecular weight in excess of about 100,000 daltons can be detected with the present method.

Thus, the present invention provides, if so desired, a method of determining the presence or absence in a sample of an antibody against an analyte of interest by an affinity assay comprising: (a) providing (i) a labeled conjugate comprising at least one label attached to a functional surrogate capable of competing effectively with an analyte of interest for a limiting amount of an antibody against the analyte, the labeled conjugate exhibiting an activity that is altered on interaction of the labeled conjugate with the antibody and which activity can be measured and related to the presence or absence of the analyte in a given sample; (b) combining the labeled conjugate with a sample suspected of containing the antibody to provide a measure of the activity; (c) measuring the activity; and (d) relating the activity to the presence or absence of the analyte in

the sample.

In a more specific embodiment, the invention allows a method to be practiced for determining the presence or absence of an analyte of interest in a sample by an affinity assay comprising: (a) providing a labeled conjugate comprising at least one label attached to a functional surrogate capable of competing effectively with an antibody against an analyte of interest for a limiting amount of the analyte, the labeled conjugate exhibiting an activity that is altered on interaction of the labeled conjugate with the analyte or with an affinity receptor for the functional surrogate and which activity can be measured and related to the presence or absence of the analyte in a given sample; (b) combining the labeled conjugate with a sample suspected of containing the analyte to provide a measure of the activity; (c) measuring the activity; and (d) relating the activity to the presence or absence of the analyte in the sample. Preferably, the interaction is at least 50% complete within about 5 minutes of the initiation of the combining step to cut down on incubation times.

In still another embodiment of the invention, a method is enabled for determining the presence or absence of an analyte of interest in a sample by an affinity assay comprising: (a) providing a labeled conjugate comprising at least one label attached to a functional surrogate of an affinity receptor for an analyte of interest, the functional surrogate capable of competing effectively with the affinity receptor for a limiting amount of the analyte, the labeled conjugate exhibiting an activity that is altered on interaction of the labeled conjugate to the analyte and which activity can be measured and related to the amount of the analyte present in a

given sample; (b) combining the labeled conjugate with a sample suspected of containing the analyte to provide a measure of the activity; (c) measuring the activity; and (d) relating the activity to the presence or absence of the analyte in the sample.

In an enzyme multiplied immunoassay format, the invention provides a method of determining the presence or absence of an analyte of interest in a sample by an homogeneous enzyme affinity assay comprising: (a) providing (i) an enzyme conjugate comprising an enzyme attached to at least one functional surrogate of an analyte of interest, the functional surrogate capable of competing effectively with the analyte for a limiting amount of an affinity receptor for the analyte, the enzyme conjugate exhibiting an activity that is altered on interaction of the enzyme conjugate to the affinity receptor and which activity can be measured and related to the amount of the analyte present in a given sample; (ii) the affinity receptor, and (iii) a substrate for the enzyme; (b) combining the enzyme conjugate, affinity receptor, and enzyme substrate with a sample suspected of containing the analyte to provide a measure of the enzyme activity; (c) measuring the enzyme activity; and (d) relating the enzyme activity to the presence or absence of the analyte in the sample.

The invention is also applicable in a fluorescence polarization assay in which the presence or absence of an analyte of interest in a sample may be determined by the steps that include: (a) providing (i) a labeled conjugate comprising a fluorescent material attached to a functional surrogate of an analyte of interest, the functional surrogate capable of competing effectively with an analyte of interest for a limiting amount of an affinity receptor for

the analyte, the labeled conjugate exhibiting an activity that is altered on interaction of the labeled conjugate with the affinity receptor and which activity can be measured and related to the presence or absence of the analyte in a given sample, and (ii) the affinity receptor; (b) combining the labeled conjugate and affinity receptor with a sample suspected of containing the analyte to provide a measure of the activity; (c) measuring the activity; and (d) relating the activity to the presence or absence of the analyte in the sample.

In a cloned enzyme donor immunoassay format, a method is provided for determining the presence or absence of an analyte of interest in a sample by an homogeneous cloned enzyme donor affinity assay comprising: (a) providing (i) a labeled conjugate comprising an enzyme donor fragment attached to a functional surrogate of an analyte of interest, the functional surrogate capable of competing effectively with the analyte for a limiting amount of an affinity receptor for the analyte, the labeled conjugate, on interaction with an enzyme acceptor fragment, exhibiting an activity that is altered in the presence of the affinity receptor and which activity can be measured and related to the amount of the analyte present in a given sample, (ii) the enzyme acceptor fragment, and (iii) the affinity receptor; (b) combining the labeled conjugate, enzyme acceptor fragment, and affinity receptor with a sample suspected of containing the analyte to provide a measure of the activity; (c) measuring the activity; and (d) relating the activity to the presence or absence of the analyte in the sample.

An affinity assay kit is also contemplated by the present invention, which comprises: (a) a labeled conjugate diposed in a

first container means, the labeled conjugate comprising at least one label attached to a functional surrogate of an analyte of interest, the functional surrogate capable of competing effectively with the analyte for a limiting amount of an affinity receptor for the analyte, the labeled conjugate capable of exhibiting an activity that is altered on interaction of the labeled conjugate with the affinity receptor and which activity can be measured and related to the amount of the analyte present in a given sample; and (b) disposed in a second container means the affinity receptor and, optionally, any substance required for the labeled conjugate to exhibit the activity, such as an enzyme substrate or an enzyme acceptor fragment.

Most importantly, in a particular embodiment of the present invention, a functional surrogate of an analyte of interest is contemplated which comprises a peptide having an immunoreactive group that allows the surrogate to compete effectively with the analyte for a limiting amount of an affinity receptor for the analyte.

It has been discovered, for instance, that certain binding peptides listed in Tables 6-16, include motifs that appear to be important to selective binding affinity. Certain of the sequences flanking the motifs may also be necessary in some cases. Specifically, for ferritin, such motifs may include: AGRALFH (SEQ ID NO:80), HGRAMFQ (SEQ ID NO:81), GGQAMFN (SEQ ID NO:82), GGSAMFS (SEQ ID NO:83), GGEALFK (SEQ ID NO:84), GGRSLFQ (SEQ ID NO:85), GGMSLFQ (SEQ ID NO:86), GGASLFQ (SEQ ID NO:87), IGASLFQ (SEQ ID NO:88), SSSALFQ (SEQ ID NO:89), SNSALFQ (SEQ ID NO:90), PQRAFFQ (SEQ ID NO:91), SINPT (SEQ ID NO:92), SINGTP (SEQ ID NO:93), GGDALFT (SEQ ID NO:94), SGGSSFW (SEQ

ID NO:95),GNTVMFQ (SEQ ID NO:96), FCGAMFC (SEQ ID NO:97), SKDSFFQ (SEQ ID NO:98), PASAFFQ (SEQ ID NO:99), HSSSLFQ (SEQ ID NO:100), NGSSLFN (SEQ ID NO:101), GGRAFFL (SEQ ID NO:102), AGRAFFR (SEQ ID NO:103), SQSS FQ (SEQ ID NO:104), HSSSLF (SEQ ID NO:105), HSSSLFQ (SEQ ID NO:106), AGAPLFQ (SEQ ID NO:107), RGNAFFK (SEQ ID NO:108), GGEVLFK (SEQ ID NO:109), GGSAAFQ (SEQ ID NO:110), GGEALFQ (SEQ ID NO:111), GGRALFA (SEQ ID NO:112), RVSTLFQ (SEQ ID NO:113), AGLALFQ (SEQ ID NO:114), HSSSFFQ (SEQ ID NO:115), SSSAFFQ (SEQ ID NO:116), PITNMFQ (SEQ ID NO:117), AGRAFFR (SEQ ID NO:118), GGDALFT (SEQ ID NO:119), GGHSFFK (SEQ ID NO:120), GGMSLFQ (SEQ ID NO:121), SGSSMFQ (SEQ ID NO:122), SSSSLFQ (SEQ ID NO:123), HSSSLFQ (SEQ ID NO:124), CRGSLFC (SEQ ID NO:125), GGMALFP (SEQ ID NO:126), GGGAMFQ (SEQ ID NO:127), RGRAMFK (SEQ ID NO:128), HSSSMFQ (SEQ ID NO:129), GGRSLFT (SEQ ID NO:130), GGASLFL (SEQ ID NO:131) or GARALFL (SEQ ID NO:132).

For the hepatitis antigen (e.g., hepatitis A, B or C, but especially B), certain motifs include HPLY (SEQ ID NO:133), HPIY (SEQ ID NO:134), GPPHL (SEQ ID NO:135), GPGPL (SEQ ID NO:136), GPGHL (SEQ ID NO:137), GPRHL (SEQ ID NO:138), VPPHL (SEQ ID NO:139), PPAHL (SEQ ID NO:140), PPPNL (SEQ ID NO:141), ARSDE (SEQ ID NO:142), LRSRE (SEQ ID NO:143), LRSAE (SEQ ID NO:144), KTVLPR (SEQ ID NO:145), GEVLPK (SEQ ID NO:146), GAVLPR (SEQ ID NO:147), GAVLAK (SEQ ID NO:148), GPKHL (SEQ ID

NO:149), GPDHL (SEQ ID NO:150), GPEHL (SEQ ID NO:151), STSSIGPLR (SEQ ID NO:152), SNTPRGPLK (SEQ ID NO:153), STTSAGPRK (SEQ ID NO:154), SGTARGPTK (SEQ ID NO:155), SLTSSGPIK (SEQ ID NO:156), RCPSDGNCY (SEQ ID NO:157) or RCPSDGLCY (SEQ ID NO:158). Accordingly, preferred binding peptides are those that include at least the primary sequence motifs depicted in Tables 1-2 and 6-16.

Furthermore, consensus sequences can be inferred from the amino acid sequences depicted in Tables 6-16. Such consensus sequences may have a particular residue conserved at a particular position. At other positions, the amino acids may vary within a particular type of residue, including but not limited to, hydrophobic amino acids (such as A, V, L, I, P, F and the like -- symbol Φ), hydrophilic residues (e.g., S, T, K, R, H, D, E, C and the like -- symbol Ψ), basic residues (e.g., K, R, H -- symbol θ), acidic residues (e.g., D, E -- symbol σ), aromatic residues (such as F, Y, W, H and the like -- symbol π) or amide containing residues (e.g., N, Q -- symbol Ω). Some residues, such as G, C or M may be considered either hydrophobic or hydrophilic. The symbol X means that a position is not conserved and may include any residue.

Hence, for a surrogate for hepatitis B antigen, certain consensus sequences can be identified, including HP(I/L)Y (SEQ ID NO:159), (SEQ ID NO:160), GPXHL (SEQ ID NO:161), (A/L)RSXE (SEQ ID NO:162), (SEQ ID NO:163), GXVLPθ (SEQ ID NO:164), STTXXGPXK (SEQ ID NO:165) or CPSDGNCY (SEQ ID NO:166). Possible consensus sequences for ferritin antigen surrogates may include GGX(A/S)LFQ (SEQ ID NO:167), (SEQ ID NO:168), SIN(P/G)TP (SEQ ID NO:169), (SEQ ID

NO:170) or GGMALFP (SEQ ID NO:171). It is important to point out, however, that other consensus sequences can be gleaned from the sequences presented in the Tables herein. Such sequences are, of course, considered part of this invention.

In addition to the labeled conjugates, the present invention also contemplates recombinant DNA constructs which comprise DNA sequences encoding a functional surrogate of an analyte of interest, the functional surrogate capable of competing effectively with the analyte for a limiting amount of an affinity receptor for the analyte. In particular, constructs comprising DNA sequences, as depicted in Tables 6-16, are particularly desired. Preferred sequences will be at least those that encode a primary sequence motif of the present invention.

Transforming vectors or expression vehicles including these constructs are also contemplated, as well as bacteriophage and viable eucaryotic and procaryotic cells transformed with such vectors or vehicles. Microorganisms can, of course, be infected with the selected bacteriophage, resulting in expression of the encoded peptides. For the production of large quantities of peptide or fusion proteins including the peptides, yeast vectors can be constructed which direct the secretion of encoded peptides into the culture medium. (See, for example, U. S. Patent No. 4,546,082, the disclosure of which is incorporated by reference herein.)

It has thus been discovered that functional surrogates of naturally occurring analytes can be obtained by a method that includes the steps of: (a) selecting an affinity receptor exhibiting a selective affinity for an analyte of interest; (b) screening a random peptide library with the affinity receptor for a binding peptide; (c)

isolating the binding peptide and identifying its structure. The peptide isolated and identified can then be synthesized and its capacity to compete with the analyte for a limiting amount of the affinity receptor verified. As discussed above, the use of a phage displayed random peptide library is particularly preferred.

The preparation and characterization of the preferred phage-displayed random peptide libraries have been described elsewhere. See, for example, Kay, B.K. et al. in *Gene* (1993) 128:59-65 and International Application No. PCT/US94/0977, for a description of the preparation of phage-displayed random peptide libraries. For a description of the libraries known as R8C, D38, and DC43, see below. In particular, by cloning degenerate oligonucleotides of fixed length into bacteriophage vectors, recombinant libraries of random peptides can be generated which are expressed at the amino-terminus of the pIII protein on the surface of M13 viral particles. (There are 3-5 copies of the pIII-fusion on the surface of each particle.) Phage display offers several conveniences: first, the expressed peptides are on the surface of the viral particles and accessible for interactions; second, the recombinant viral particles are stable (i.e., can be frozen, exposed to pH extremes); third, the viruses can be amplified; and fourth, each viral particle contains the DNA encoding the recombinant genome. Consequently, these libraries can be screened by isolating viral particles that bind to targets. The isolates can be grown up overnight, and the displayed peptide sequence responsible for binding can be deduced by DNA sequencing.

These libraries have approximately $>10^8$ different recombinants, and nucleotide sequencing of the inserts suggests that

the expressed peptides are indeed random in amino acid sequence.

6. Examples

The following Examples are provided to assist the reader further, which Examples describe selected materials, compositions, and methods for use in particular embodiments and which are illustrative of the invention, as a whole.

Examples of materials used in the methods of the invention are set forth below.

Chemical Reagents

10xPBS; Dulbecco's PBS x 10 (JRH Biosciences, Lenexa, KS, Cat # 59331-78P)

PBS; phosphate buffered saline (1-10 dilution in water of Dulbecco's 10x PBS)

BSA; bovine serum albumin (Sigma, St Louis, MO; Cat # A7906)

Tween 20; polyoxyethylene sorbitan monolaurate (Sigma, St Louis, MO; Cat # P1379)

PBS/BSA; PBS with 1% BSA

PBT; PBS with BSA (1%) & Tween 20 (0.05%)

X-Gal; X-Gal (Jersey Lab. Supply; Livingston, NJ; Cat # X266)

DMF; dimethyl formamide (Sigma, St Louis, MO; Cat # D4254)

X-Gal solution; 2% X-Gal in DMF

TMB; tetramethyl benzidine substrate (KPL, Gaithersburg, MD; Cat # 50-76-00)

2xYT broth;

Streptavidin-coated microtiter plates; Reactibind (Pierce, Rockford, IL, Cat # 15120)

Microtiter plates (Immulon 4, Dynatech, Chantilly, VA; Cat # 011-010-3855)

DH5xF': *E. coli* cells

IPTG; isopropyl-ß-D-thiogalactopyranosidase (Jersey Lab Supply, Livingston, NJ; Cat # I555)

IPTG solution; 100 mM IPTG in water

SM buffer

G6PDH; glucose 6 phosphate dehydrogenase (Sigma, St. Louis, MO; Cat # G5760)

NHS; N-hydroxysuccinimide (Sigma, St. Louis, MO; Cat # H7377)

DCC; dicyclohexylcarbodiimide (Sigma, St. Louis, MO; Cat # D3128)

DMSO; dimethylsulfoxide (Sigma, St. Louis MO; Cat # D5879)

Tris; TRIZMA base (Sigma, St Louis, MO; Cat # T8524)

G6P; glucose 6 phosphate, sodium salt (Sigma, St. Louis, MO; Cat # G7879)

Carbitol; diethyleneglycol monoethylether (Sigma, St. Louis, MO.; Cat # D1265)

NADH; nicotinamide adenine dinucleotide, reduced form (Sigma, St. Louis, MO; Cat # N6005)

PEG; polyethylene glycol ave. MW 8000 (Sigma, St. Louis, MO; Cat # P2139)

NaCl; sodium chloride (Sigma, St. Louis, Mo.; Cat # S9625)

PEG/NaCl; 20% PEG 8000 in 2.5 M NaCl

<u>Immuno Reagents</u>

Affinity purified goat anti-HBsAg (OEM Concepts, Toms River,

NJ: Cat # G5-V18)

Affinity purified sheep anti-ferritin (The Binding Site, San Diego, CA: Cat # AU055)

Monoclonal mouse anti-HBsAg (OEM Concepts, Toms River, NJ; Cat # M2-V18)

Goat anti-mouse IgG HRP conjugate (OEM Concepts, Toms River, NJ.;

Cat # G5-MG16-2)

Rabbit anti-goat IgG HRP conjugate (OEM Concepts, Toms River, NJ:

Cat # R5-GG10-2)

Rabbit anti-sheep IgG HRP conjugate (OEM Concepts, Toms River, NJ;

Cat #R5-SG10-2)

Normal non-immune mouse IgG (OEM Concepts, Toms River, NJ: Cat # M6-G10)

Normal non-immune goat IgG (OEM Concepts, Toms River, NJ; Cat # G6-G10)

Normal non-immune sheep IgG (OEM Concepts, Toms River, NJ; Cat # S8-G10)

rHBsAg: recombinant HBsAg AY antigen (OEM Concepts, Toms River, NJ;

Cat # H7-V57)

Ferritin antigen (OEM Concepts, Toms River, NJ; Cat # H6-MO5)

Rabbit anti-M13 HRP conjugate (Pharmacia, Piscataway, NJ, Cat # 27-9402A)

Phage Display Libraries

The following Libraries were used for biopanning with mouse anti-HBsAg and goat anti-HBsAg: R8C and R26.

For biopanning with sheep anti-ferritin, the following libraries were used: D38; DC43; and R26.

6.1. Preparation of a Phage Display Random Peptide Library

6.1.1. General Synthesis and Assembly of Oligonucleotides

Random sequence oligonucleotide inserts flanked by selected cloning sites were synthesized with an applied Biosystems 380a synthesizer (Foster City, CA), and the full-length oligonucleotides were purified by HPLC.

Five micrograms of each of the pair of oligonucleotides were mixed together in buffer (10 mM Tris-HCl, pH 8.3, 15 mM KCl, 0.001% gelatin, 1.5 mM magnesium chloride), with 0.1 % Triton X-100, 2 mM dNTP's, and 20 units of Taq DNA polymerase. The assembly reaction mixtures were incubated at 72 °C for 30 seconds and then 30 °C for 30 seconds; this cycle was repeated 60 times. It should be noted that the assembly reaction is not PCR, since a denaturation step was not used. Fill-in reactions were carried out in a thermal cycling device (Ericomp, LaJolla, CA) with the following protocol: 30 seconds at 72 °C, 30 seconds at 30 °C, repeated for 60 cycles. The lower temperature allows for annealing of the six base complementary region between the two sets of the oligonucleotide pairs. The reaction products were phenol/chloroform extracted and ethanol precipitated. Greater than 90% of the nucleotides were found to have been converted to double stranded synthetic oligonucleotides.

After resuspension in 300 μL of buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE buffer), the ends of the oligonucleotide fragments were cleaved with *Xba* I and *Xho* I (New England BioLabs, Beverly, MA) according to the supplier's

recommendations. The fragments were purified by 4% agarose gel electrophoresis. The band of correct size was removed and electroeluted, concentrated by ethanol precipitation and resuspended in 100 μL TE buffer. Approximately 5% of the assembled oligonucleotides can be expected to have internal *Xho* I or *Xba* I sites; however, only the full-length molecules were used in the ligation step of the assembly scheme. The concentration of the synthetic oligonucleotide fragments was estimated by comparing the intensity on an ethidium bromide stained gel run along with appropriate quantitated markers. All DNA manipulations not described in detail were performed according to Sambrook, et al., *infra*.

To demonstrate that the assembled enzyme digested oligonucleotides could be ligated, the synthesized DNA fragments were examined for their ability to self-ligate. The digested fragments were incubated overnight at 18 °C in ligation buffer with T4 DNA ligase. When the ligation products were examined by agarose gel electrophoresis, a concatamer of bands was visible upon ethidium bromide staining. As many as five different unit length concatamer bands (i.e., dimer, trimer, tetramer, pentamer, hexamer) were evident, suggesting that the synthesized DNA fragments were efficient substrates for ligation.

6.1.2. <u>Construction of Vectors</u>

The construction of the M13 derived phage vectors useful for expressing a random peptide library has been recently described (Fowlkes, D. et al. *BioTech.* (1992) 13:422-427). To express the library, an M13 derived vector, m663, was constructed

as described in Fowlkes. The m663 vector contains the pIII gene having a c-myc-epitope, i.e., as a stuffer fragment, introduced at the mature N-terminal end, flanked by *Xho* I and *Xba* I restriction sites (See also, Figure 1 of Fowlkes).

6.1.3. Expression of the Random Peptide Library

The synthesized oligonucleotides were then ligated to *Xho* I and *Xba* I double-digested m663 RF DNA containing the pIII gene (Fowlkes) by incubation with ligase overnight at 12 °C. More particularly, 50 ng of vector DNA and 5 ng of the digested synthesized DNA and were mixed together in 50 μL ligation buffer (50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 20 mM DTT, 0.1 mM ATP) with T4 DNA ligase. After overnight ligation at 12 °C, the DNA was concentrated by ethanol precipitation and washed with 70% ethanol. The ligated DNA was then introduced into *E. coli* (DH5αF'; GIBCO BRL, Gaithersburg, MD) by electroporation.

A small aliquot of the electroporated cells was plated and the number of plaques counted to determine that 10^8 recombinants were generated. The library of *E. coli* cells containing recombinant vectors was plated at a high density (~400,000 per 150 mM petri plate) for a single amplification of the recombinant phage. After 8 hr, the recombinant bacteriophage were recovered by washing each plate for 18 hr with SMG buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.05% gelatin) and after the addition of glycerol to 50% were frozen at -80 °C. The library thus formed had a working titer of ~2 x 10^{11} pfu/ml.

6.2. Preparation of R8C Library

Referring now to Figure 7, two oligonucleotides were synthesized on an Applied Biosystems Model 380a machine with the sequence 5'-TGACGTCTCGAGTTGTNNKNNKNNKNNKNNKNNKNNKNNKTGTGGATCTAGAAGGATC-3' (SEQ ID NO:172) and 5'-GATCCTTCTAGATCC-3' (SEQ ID NO:173), where N is an equimolar ratio of deoxynucleotides A, C, G, and T, and K is an equimolar ratio of G and T. Fifty pmol of each oligonucleotide was incubated at 42 °C for 5 min, then 37 °C for 15 minutes in 50 µL of Sequenase™ buffer (U.S. Biochemicals, Cleveland, OH) with 0.1 µg/µL acetylated BSA, and 10 mM DTT. After annealing, 10 units of Sequenase™ (U.S. Biochemicals) and 0.2 mM of each dNTP were added and incubated at 37 °C for 15 min. The sample was then heated at 65 °C for 2 hr, digested with 100 units of both *Xho* I and *Xba* I (New England BioLabs, Beverly, MA), phenol extracted, ethanol precipitated, and resolved on a 15% non-denaturing polyacrylamide gel. The assembled, digested fragment was gel purified prior to ligation. The vector, m663 (Fowlkes, D. et al. *Biotech.* (1992) 13:422-427), was prepared by digestion with *Xho* I and Xba I, calf alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) treatment, phenol extracted, and purified by agarose gel electrophoresis. To ligate, 20 µg vector was combined with 0.2 µg insert in 3 mL with T4 DNA ligase (Boehringer Mannheim), according to the manufacturer. After removal of the protein and buffer by phenol extraction and ethanol precipitation, the ligated DNA was electroporated into XL1-Blue E. coli (Stratagene, San Diego, CA) and plated for eight hours at 37 °C.

To recover the recombinant phage, the top agar was collected with a spatula, mixed with an equal volume of 100 mM NaCl, 10 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.5), and disrupted by two passes through an 18-gauge syringe needle. The bacterial cells were removed by centrifugation, and phage particles were collected by polyethylene glycol precipitation and stored at -70 °C in 25% glycerol. The library had 10^8 total recombinants and a working titer of 6 x 10^{13} pfu/mL.

Members of the library were checked for inserts by the polymerase chain reaction (Saiki, et al. *Science* (1988) 239:487-491). Individual plaques on a petri plate were touched with a sterile toothpick and the tip was stirred into 2xYT with F^+ *E. coli* bacteria and incubated overnight at 37 °C with aeration. Five microliters of the phage supernatant were then transferred to new tubes containing buffer (67 mM Tris-HCl, pH 8.8/10 mM β-mercaptoethanol/16.6 mM ammonium sulfate/6.7 mM EDTA/50 μg bovine serum albumin per mL), 0.1 mM deoxynucleotide triphosphates, and 1.25 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) with 100 pmoles of oligonucleotide primers. The primers flanked the cloning site in the pIII gene of m663 (5'-TTCACCTCGAAAGCAAGCTG-3' (SEQ ID NO:174) and 5'-CCTCATAGTTAGCGTAACG-3' (SEQ ID NO:175)). The assembly reactions were incubated at 94 °C for 1 min, 56 °C for 2 min, and 72 °C for 3 min; this cycle was repeated 24 times. The reaction products were then resolved by electrophoresis on a NuSieve 2.0% agarose gel (FMC, Rockland, ME). Gels revealed that for 20 plaques tested, all were recombinant and had single inserts of the expected size.

The R26, D38, and DC43 libraries were prepared similarly based on the schematic provided in FIGS. 8, 9, and 10.

6.3. Biopanning

Biopanning was carried out on microtiter wells that had been coated with 100μL of immune antiserum (e.g., mouse monoclonal anti-HBsAg) diluted in PBS to 5 ug/mL. The wells were blocked with 100 μL PBS/BSA overnight at 4 °C, washed 3x with 200 μL PBS/BSA, then banged dry on paper towels.

Phage libraries to be panned were diluted in PBS to approximately 10^{10} to 10^{11} pfu per 40 μL. Diluted phage library (40μL) was added to each well, the plate covered, and allowed to incubate on a rocker for 1 hr at room temperature.

The contents of the wells were dumped, and the wells washed 10x with 200 μL PBS/BSA, then banged dry on paper towels. Bound phage was then eluted by adding 50 μL of 0.05 M glycine pH 2.0 to each well and incubating for 5 min at room temperature on the rocker.

50 μL of 0.2 M Phosphate pH 7.6 was added to a separate tube and, after the 5 minute incubation, the 50 μL of eluted phage (in the glycine pH 2.0) was added to the 0.2 M phosphate pH 7.6.

6.4. Isolation and Characterization of Antibody Binding Phage from Libraries

To illustrate the invention, antibody (monoclonal mouse anti-HBsAg or affinity purified polyclonal goat anti-HBsAg or affinity purified sheep anti-human ferritin) was used to biopan the libraries. Three (3) rounds of biopanning were performed, and

binders were amplified once between the 1st and 2nd rounds.

Binders from the 3rd round of panning were grown on agar plates. Routinely 30 individual plaques were picked manually, grown in liquid culture overnight at 37 °C and assayed in the M13 phage ELISA to determine specific binding to antibody.

DNA from positive clones was isolated and sequenced. The sequences were then examined manually and any consensus motifs determined.

6.5. ELISA for Phage M13

Specific phage binders were confirmed with an ELISA for M13. The assay was performed on phage cultures that had been grown overnight from the 30 plaques that had been picked individually after the 3rd round of biopanning.

Alternate rows of a microtiter plate were coated with 50 μL of immune antibody at 5 μg/mL in PBS (e.g., mouse anti HBsAg), appropriate non-immune IgG at 5 μg/mL in PBS, and PBS/BSA for one hour at room temperature. The solutions were dumped and the wells blocked for a minimum of one hour at room temperature with 200 μL PBS/BSA. The solutions were dumped, the wells washed 3x with 200 μL PBS/BSA, and then banged dry on paper towels.

Phage to be assayed (phage growth medium supernatant from a 6h liquid growth) was diluted 1-100 in PBS/BSA, and 100 μL of this dilution was added to each of 6 wells - 2 coated with immune IgG, 2 coated with non-immune IgG, and 2 coated with BSA; i.e., each phage sample was assayed in duplicate, with non-specific binding (NSB) controls of non-immune serum and BSA.

The plate was incubated at room temperature for 1 hour,

then washed 3x with 200 μL PBS/BSA and banged dry on paper towels. 100 μL anti-M13 HRP (diluted 1:8000 in PBS/BSA) was added to each well, the plate incubated for 1 hour at room temperature, then washed 3x with 200 μL PBS/BSA and banged dry on paper towels.

100 μL TMB substrate was added to each well and the blue color allowed to develop for about 5 minutes. The plate was then read at 620 nm on the SLT 340 ATTC or Magnetic Devices V max Microplate Reader.

Positive phage were identified as binding to the antibody coated wells, but not the wells coated with non-immune IgG or BSA.

6.6. Phage Amplification

Phage amplification on plates and in liquid was carried out as described, McConnell, SJ., Uveges, AJ., & Spinella, DG. *Biotechniques* (1995) 18:803-806.

When amplification was used in between rounds 1 and 2 of biopanning, debris was removed from solution by centrifugation at 1000 rpm for 10 minutes at 4 °C, then phage was precipitated with 0.2 vol PEG/NaCl for 2h on ice. Supernatant was removed after centrifugation at 10,000 rpm for 15 minutes at 4 °C and phage dissolved in 100 μL of PBS/BSA and transferred to a microtube. The solution was clarified by centrifugation (10,000 rpm, 10 minutes) and used immediately.

6.7. DNA Sequencing

DNA sequencing of phage clones confirmed positive from

the M13 ELISA was used to determine the amino acid sequence of the random peptide insert.

Single stranded DNA template was prepared using the Dynabeads [R] lacZ ssM13 Purification Kit (Applied Biosystems/Perkin Elmer, Foster City, CA; Cat # 401436) according to the protocol provided by the manufacturer.

DNA sequencing was performed on a model 373A DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) using the Prism ™ Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems/Perkin Elmer, Foster City, CA; Cat # 401384) according to the protocol provided by the manufacturer.

PC/GENE Release 6.7 (Intelligenetics, Mountain View, CA) was used to analyze the DNA sequence, and determine the deduced amino acid sequence of the random peptide insert.

6.7.1. Anti-HBsAg Binders. Mouse Monoclonal Anti-HBsAg

Libraries giving positive phage binders when panned with monoclonal anti-HBsAg were R26 and R8C. The sequences and consensus motifs are shown in Table 4. From a total of 26 unique (non-sibling) sequences examined, four consensus motifs could be recognized, with only three sequences not containing any of the four motifs. None of the motifs appeared to match any portion of the HBsAg primary sequence, hence these motifs were regarded as mimetopes.

6.7.2. Affinity Purified Goat Polyclonal Anti-HBsAg

Libraries giving positive phage binders when panned

with goat anti-HBsAg were R26a, b, c & d, and R8C. The sequences and consensus motifs are shown in the Table 4. From a total of 12 unique (non-sibling) sequences examined, two consensus motifs could be recognized. The four M13 ELISA positive phage from the R8C Library failed to give any consensus motif that could be recognized.

Both of the motifs appeared to match a portion of the HBsAg primary sequence, hence these motifs were regarded as epitopes. Only the R8C Library failed to produce any consensus motif from the M13 ELISA confirmed positive binders.

6.8. Solid Phase Peptide Synthesis

Consensus peptide motifs deduced from DNA sequencing were assembled using a modification of Merrifield's solid phase method (Merrifield, 1963) using either standard HBTU chemistry on a Model 430A Peptide Synthesizer (Perkin Elmer/Applied Biosystems, Foster City, CA) or using the same chemistry on a Symphony Multiple Peptide Synthesizer (Rainin/PTI, Woburn, MA) Resin used for assembly was Tentagel S RAM (Tubingen, Germany).

9-Fluorenylmethoxycarbonyl (Fmoc) derivatives of amino acids were used throughout, with side chains blocked by t-butyl type moieties. The same HBTU chemistry was used to add biotin at the amino terminus of some peptides. Stepwise reaction efficiencies were monitored by ninhydrin (Kaiser, 1970), and were typically >95%.

Resin cleavage and side chain deblocking were performed simultaneously for 120 minutes at room temperature using reagent

K (King, 1990). Multiple washes with t-butyl methyl ether were performed after cleavage to remove scavengers.

Peptides were desalted and purified via preparative reverse phase HPLC before lyophilization and storage at room temperature. Amino acid analyses (AAA) were carried out on a Beckman System Gold (Beckman, Fullerton, CA) after vapor phase HCl hydrolysis (Meltzer, 1987). Mass Spectroscopy (FAB-MS) was performed by M Scan (West Chester, PA.).

6.9. <u>Oxidation of Peptides</u>

Peptides designed to contain a disulfide bond were oxidized immediately. Crude peptide was dissolved at 0.5 mg/ml in 50 mM sodium phosphate, pH 8.0, with 1% acetonitrile added as antimicrobial. The mixture was stoppered loosely with glass wool and allowed to stir gently on a magnetic stirrer in contact with air. Oxidation was allowed to proceed for 12-36 hours, and the reaction was monitored with 5,5'-dithio-bis(nitrobenzoic acid) (Deakin, 1963) to determine the end-point.

6.10. <u>Titration of Peptide and Primary Ab by ELISA</u>

Reaction of synthesized peptides with antibody was detected using a microtiter plate based ELISA. Varying amounts of synthetic peptide were immobilized either via attached biotin (to streptavidin coated plates) or chemically. Binding of varying amounts of added antibody to the immobilized synthetic peptide was detected with HRP conjugated 2nd antibody. As shown in FIG. 1, the more synthetic peptide is present in the microtiter plate, the greater the response from added antibody reagents. Hence, the

synthetic peptides of the present invention exhibit the selective binding characteristics expected of a functional surrogate for naturally occurring hepatitis B surface antigen. Similar results are shown in FIGS. 3 and 5.

6.10.1 Immobilization of Biotinylated Peptides

Biotinylated peptide was diluted in PBT and coated onto streptavidin plates at 4°C overnight. 100µL of diluted solution was used per well. The contents were dumped, the plate washed 3x with 200 µL PBS/BSA/T, and banged dry on paper towels.

6.10.2. Immobilization of Non-Biotinylated Peptides With Carbodiimide

Non-biotinylated peptides were immobilized onto microtiter plates using CDI by the method of Dagensis, P., in *Anal. Biochm.* (1994) 222:149-156. Irrespective of the immobilization procedure, the subsequent assay was carried out as follows:

6.10.3. Assay Procedure

Primary antibody (e.g., mouse anti-HBsAg) was diluted in PBT. 100 µL of each dilution was added to appropriate wells and incubated at room temperature for one hour on a rocker. The contents were dumped, the plate washed 3x with 200 µL PBS/BSA/T, and banged dry on paper towels.

Appropriate 2nd Ab HRP conjugate (e.g., goat anti-Mouse HRP) was diluted 1:15,000 in PBT, 100 µL added to each well, and incubated for one hour at room temperature on a rocker. The contents were dumped, the plate washed 3x with 200µL

PBS/BSA/T, and banged dry on paper towels.

100 μL of TMB substrate was added to each well and allow to develop for 15-30 minutes at room temperature. The plate was then read at 650nm on the SLT 340 ATTC or Magnetic Devices V max Microplate Reader.

6.11. ELISAs for Antigen Using Immobilized Antigen or Binding Peptide

The ability of a synthetic binding peptide to act as a functional surrogate of the natural antigen was demonstrated by constructing competitive and sequential ELISAs for antigen using either immobilized antigen or synthetic peptide bound to a solid phase. The resulting competitive binding profiles for three selected synthetic peptides are presented in FIGS. 2, 4 and 6. As illustrated in these figures, the functional surrogates of the present invention can compete effectively for limited antibody in the presence of natural whole antigen.

6.11.1. Immobilization of Antigen, Biotinylated and Non-Biotinylated Peptides

Whole antigen was diluted in PBS and immobilized onto microtiter plates using passive adsorption at 4 °C overnight. Biotinylated peptides were immobilized on streptavidin coated plates, and non-biotinylate peptides were covalently bound using CDI, as previously described.

6.11.2. Sequential ELISA

Antigen and antibody were diluted in PBT. Equal volumes of each antigen concentration and antibody solution were mixed in separate glass 12 x 75 mm tubes and incubated at room temperature for 90 minutes. Triplicate 100 µL portions of each mixture were added to appropriate wells of a microtiter plate and incubated at room temperature for 2.5h on a rocker.

The subsequent procedure was used irrespective of immobilization method or whether the assay was competitive or sequential.

The contents were dumped, the plate washed 3x with 200 µL PBS/BSA/T, and banged dry on paper towels.

The appropriate second Ab HRP conjugate was diluted 1:15000 in PBT, and 100 µL of the diluted solution was added to each well. The resulting mixture was incubated for one hour at room temperature on a rocker. The contents were the dumped, the plate washed 3x with 200 µL PBS/BSA/T, and banged dry on paper towels.

100 µL of TMB substrate was added to each well and the reaction was allowed to develop for 30 minutes at room temperature. The plate was then read at 650 nm on the SLT 340 ATTC or Magnetic Devices V max Microplate Reader.

6.12. Conjugation of Peptide to G6PDH

The peptide N-hydroxysucccinimide ester (0.1 M) was prepared in dimethyl formamide with equimolar concentrations of NHS (0.1 M) and DCC (0.1 M). The active ester was then coupled to G6PDH as described by Oellerich (1986).

The functional surrogates are normally bonded to the enzyme directly through a single or double bond, or indirectly through a linking group. The functional groups of the enzyme which are available for linking are amino (including guanidino), hydroxy, carboxyl, mercapto, and activated aromatic groups of imidazole.

The binding peptides have a great diversity of functional groups available for coupling; additionally, routine modifications of these functionalities may be made to facilitate the conjugation, e.g., conversion of keto to hydroxy, or olefin to aldehyde or carboxylic acid.

Where a linking group is employed for bonding the functional surrogate to the enzyme label, normally the linker will be attached to the peptide to provide a means for coupling of the peptide to the enzyme. This conjugation may be achieved in a single step or may require multiple steps, including blocking and unblocking of active sites of the peptide other than those involved in providing the linking group.

Where the enzyme is to be linked through a carboxyl group of the functional surrogate or a linker bonded to the functional surrogate, either esters or amides will be prepared. The functional surrogate may be bonded to any of the linking groups which are appropriate to provide a link between the functional surrogate and the alcohol or amine group of the enzyme to form the ester or amide group, respectively. When the enzyme has an activated aromatic ring, the functional surrogate may be bonded to an aromatic diazonium salt to provide the desired bridge.

When bonding a functional surrogate through a linking

group to an enzyme, the bonds formed must be stable under assay conditions and the conditions used for carrying out the coupling reactions must not result in an inactive enzyme conjugate. Additionally, the enzyme must not prevent binding of receptor to functional surrogate.

For instance, if the functional surrogate has an amino group the amino may be derivatized to alpha-bromoacetamide. This group can then form a C-N bond to an amino acid of an enzyme that has a free amino group (such as lysine). If the functional surrogate has a keto group, the carbonyl may be condensed directly with an amine group of the enzyme, or the O-carboxy methyloxime may be prepared with O-carboxymethyl hydroxylamine. A mixed anhydride, with isobutyl chloroformate is formed, which can then form the carboxamide with the amino group of a lysine. Where a carboxyl group is present in the functional surrogate, this group may be derivatized to the monoamide of phenylenediamine. The intermediate can then be diazotized to form a diazo salt suitable for coupling to a tyrosine in the enzyme.

Also a hydroxyl group is present in the functional surrogate it can be reacted with succinic anhydride to form a monoester. The free carboxy group can then be used to prepare the mixed anhydride, which in turn can be reacted with an amino group in the enzyme.

Where an amino group is present in the functional surrogate this may be reacted with maleic anhydride to give the maleimide. The maleimide may then be reacted with cysteine in the enzyme to give a 3-thiosuccinimide.

6.13. Procedure for EMIT Assay

Using a set amount of conjugate, a solution of antibody is titrated to give approximately 40-60% inhibition of activity. In the presence of antigen, the inhibitory effect of added conjugate on antibody activity is proportional to the amount of antigen present. Thus, a dose response curve of G6PDH activity vs antigen concentration can be obtained.

6.14 Procedure for Testing Peptide-G6PDH Conjugate

G6PDH activity is measured by the rate of conversion of NAD to NADH as measured by increase in absorbance at 340 nm at 37 °C. The dilution of conjugate for use in the EMIT assay is determined from the results.

Peptide-G6PDH conjugate (conjugate) is diluted 1:10, 20, 40, 80, 160, 320 and 640 in a buffer of 0.218 M Tris pH 8.0 containing 1 g/L BSA and 7g/L Glucose 6 phosphate.

25 µL portions of diluted conjugate are added to appropriate wells of a microtiter plate. 100 µL of buffer (0.013 M Tris pH 6.0 containing 1 g/L BSA, 20 g/L NaCl, 1 mL/L Tween 20) is added to each well and the mixture brought to reaction temperature of 37 °C. 25 µL of 0.013 M Tris pH 6.0 containing 1 g/L BSA, 20g/L NaCl, 1 mL/L Tween 20, and 16.62 g/L NAD prewarmed to 37 °C is added to each of the wells. The mixtures are shaken for 1 minute then G6PDH activity is monitored by measuring the absorbance at 340 nm every 30 seconds for 3 minutes at 37 °C.

The dilution of conjugate giving an activity of between 200-250 mA/min is used for the EMIT assay.

6.14.1. Example: Conjugate of Peptide SEQ. ID. NO. 26 and G6PDH, designated EC1.

EC1 is diluted 1:10, 20, 40, 80, 160, 320 and 640 in a buffer of 0.218 M Tris pH 8.0 containing 1 g/L BSA and 7 g/L glucose-6-phosphate.

25 μL portions of diluted conjugate are added to appropriate wells of a microtiter plate. 100 μL of buffer (0.013 M Tris pH 6.0 containing 1 g/L BSA, 20g/L NaCl, 1 mL/L Tween 20) is added to each well and the mixture brought to reaction temperature of 37 °C. 25μL of 0.013M Tris pH 6.0 containing 1g/L BSA, 20g/L NaCl, 1mL/L Tween 20, and 16.62 g/L NAD prewarmed to 37 °C is added to each of the wells. The mixtures are shaken for 1 minute then G6PDH activity is monitored for 3 minutes by measuring the absorbance at 340 nm every 30 seconds for 3 minutes at 37 °C.

Results:

Dilution EC1: 1:	Rate mA/min
10	800
20	393
40	218
80	123
160	63
320	35
640	18

A dilution of 1:40 was used for inhibition experiments with antibody and antigen (EMIT assay).

6.15. Determination of Amount of Antibody to Use

Commercially obtained antibody is diluted 1:10, 20, 40, 80, 160, 340 and 320 in a buffer of 0.013 M Tris pH 6.0 containing 1

g/L BSA, 20 g/L NaCl, 1 mL/L Tween 20. 100 μL portions of each dilution are added to wells of a microtiter plate. To each well is added 25 μL of diluted conjugate - dilution determined from activity experiments to give a rate of approx. 200-250 mA/min -- and the mixtures are incubated for 5-10 minutes at room temperature. The mixtures are then warmed to 37 °C and 25 μL of 0.013 M Tris pH 6.0 containing 1 g/L BSA, 20 g/L NaCl, 1 mL/L Tween 20, and 16.62 g/L NAD prewarmed to 37 °C are added to each of the wells. The mixtures are shaken for 1 minute, then G6PDH activity is monitored for by measuring the absorbance at 340 nm every 30 secs for 3 minutes at 37 °C.

The dilution of antibody giving an inhibition of 40-60% of G6PDH activity is used for the EMIT assay.

6.15.1. Example

Goat anti-HBsAg is diluted 1:10, 20, 40, 80, 160, 340 and 320 in a buffer of 0.013 M Tris pH 6.0 containing 1 g/L BSA, 20 g/L NaCl, 1 mL/L Tween 20. 100 μL portions of each dilution are added to wells of a microtiter plate. To each well is added 25 μL of conjugate EC1 diluted 1:40 and the mixtures are incubated for 5-10 minutes at room temperature. The mixtures are then warmed to 37 °C and 25 μL of 0.013 M Tris pH 6.0 containing 1 g/L BSA, 20 g/L NaCl, 1 mL/L Tween 20, and 16.62 g/L NAD prewarmed to 37 °C are added to each of the wells. The mixtures are shaken for 1 minute then G6PDH activity is monitored by measuring the absorbance at 340 nm every 30 secs for 3 minutes at 37 °C.

Results:

Dilution of Ab: 1:	Rate mA/min
10	125
20	105
40	135
80	165
160	200
320	217
640	215
no Ab	220

Dilution of goat anti-HBsAg to use for antigen experiments. 1:30.

6.16. Dose Response to Antigen

Antibody dilution used is determined from the section, above. Conjugate dilution used is determined from previous experiments as used in the section, above.

10-15 μL of sample containing antigen is incubated for 10-15 minutes with 100ul of diluted antibody and 25μL of 0.013 M Tris pH 6.0 containing 1 g/L BSA, 20 g/L NaCl, 1 mL/L Tween 20, and 16.62 g/L NAD. The mixtures are then warmed to 37 °C and 25 μL diluted conjugate pre-warmed to 37 °C is added. The mixtures are shaken for 1 minute then G6PDH activity is monitored for 3 minutes by measuring the absorbance at 340 nm every 30 secs for 3 minutes at 37 °C.

The resulting dose response curve shows an increase in G6PDH activity (as a rate measurement: mA/min at 340 nm) proportional to the concentration of antigen added in the sample.

6.16.1. Example

10-15 μL of sample containing 0-200 ng/mL of rHBsAg antigen is incubated for 10-15 minutes with 100 μL of goat anti-HBsAg antibody (diluted 1:30) and 25 μL of 0.013 M Tris pH 6.0 containing 1 g/L BSA, 20 g/L NaCl, 1 mL/L Tween 20, and 16.62 g/L NAD. The mixtures are then warmed to 37 °C and 25 μL conjugate EC1 diluted 1:40 and pre-warmed to 37 °C are added. The mixtures are shaken for 1 minute, then G6PDH activity is monitored by measuring the absorbance at 340 nm every 30 secs for 3 minutes at 37 °C.

Results:

Conc. HBsAg ng/mL	Rate mA/min
0	100
3	112
6	122
12	140
25	165
50	185
100	205
200	220

An increasing rate of G6PDH enzyme activity is seen with increasing amounts of rHBsAg in the sample.

6.17 Another Example of an EMIT Assay

6.17.1. Conjugation of Binding Peptides to Enzyme Label

SEQ. ID. NO. 35 H_2N-LPGPPHLS-COOH FW 816

SEQ. ID. NO. 37	H_2N-C-oK-LPGPPHLS-$CONH_2$ | H_2N-C-oK-LPGPPHLS-$CONH_2$	FW 2100
G6PDH	FW 120,000	
G6P	FW 282	
NADH	FW 709	
2-iminothiolane	FW 138	
SPDP	FW 312	

The peptide of SEQ. ID. NO. 37 (31 mg) is prepared by air oxidation of the reduced form of the peptide in Kpi, pH 8 (reduction is effected by treatment with 1 mM tributyphosphine). Completion of oxidation is confirmed from a negative Ellman's test. Oxidized peptide is purified by reverse-phase HPLC, then lyophilized.

An amount (1 mg) of the synthetic peptides, SEQ. ID. NO. 35 (ca. 1 umole) and SEQ. ID. NO. 37 (ca. 500 μmole), is dissolved in 200 μL PBS, 1 mM EDTA (PBSE - PBS containing 1 mM EDTA). The peptides are then thiolated using a 25-fold molar excess of a 100 mM (3.5 mg/250 μL) solution of Traut's reagent in PBSE (50 μL and 25 μL, respectively). After allowing the resulting mixture to incubate in the dark for 1 hour at room temperature, the mixtures are exchanged into PBSE through a Sephadex G10 gel filtration column (Pharmacia, Piscataway, NJ).

Separately, extensive dialysis of commercially obtained G6PDH enzyme in PBS provided approximately 1.2 mg G6PDH enzyme for dilution to a working concentration of 1 mg/mL (8 μM, 390 U/mg). To 1.0 mL of this enzyme solution, G6P and NADH is added from fresh stocks (500 mM in PBSE) to provide a final concentration of 10 mM each. The enzyme is then allowed to react

with 10-fold molar excess (80 μL) of a fresh 1 mM (0.5 mg/1.5 mL) solution of N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pierce, Rockford, IL) in carbitol. After a reaction period of 1h at room temperature, small molecules are removed by desalting on G10 in PBSE.

Labeled conjugates are formed by allowing appropriate amounts of the thiolated peptides to react with the activated enzyme in the refrigerator overnight.

Both preparations of conjugates are then harvested, filtered through a 1.0 um PFTE syringe filter, and brought to a volume of 3 mL by addition of an appropriate amount of Tris buffer. The resulting solutions of the labeled conjugates are then used in subsequent experiments as described further below.

6.17.2. Preparation of Diluted Solutions of Labeled Conjugates

Conjugates of peptides, SEQ. ID. NO. 37 and SEQ. ID. NO. 35 with G6PDH are designated EC4 and EC5, respectively.

Lyophilized samples of conjugates EC4 and EC5, as prepared above, are diluted 1:100, 200, 400, 800, 1600, 3200 and 6400 in a buffer of 0.218 M Tris (pH 8.0), containing 1 g/L BSA and 6 g/L glucose-6-phosphate.

Diluted solutions of each G6PDH-peptide conjugate (in 25 μL portions) are added to appropriate wells of a microtiter plate. Then, 100 μL of buffer (0.013 M Tris (pH 6.0), containing 1 g/L BSA, 20 g/L NaCl, 1 mL/L Tween 20) are added to each well. The resulting mixture is allowed to warm to the reaction temperature of 37 °C.

Next, 25 μL of 0.013 M Tris (pH 6.0), containing 1 g/L BSA,

20 g/L NaCl, 1 mL/L Tween 20, and 16.62 g/L NAD (substrate for G6PDH) prewarmed to 37 °C are added to each of the wells. The mixtures are shaken for 1 minute, then G6PDH activity is monitored by measuring the absorbance at 340 nm every 30 seconds for 3 minutes at 37 °C.

The results are presented in the following Table.

Table 4. G6PDH Enzyme Activity

RATE; mA/min at 340 nm						
	EC4			EC5		
DILUTION 1:	1	2	MEAN	1	2	MEAN
BLANK	0	0	0	0	1	0
100	221	214	217	333	312	322
200	120	117	118	160	170	165
400	65	64	64	90	91	90
800	34	34	34	48	48	48
1600	17	18	17	25	31	28
3200	10	13	11	13	14	13
6400	5	5	5	7	7	7

Consequently, to provide a signal level convenient for detection, a dilution of 1:150 is chosen for conjugate EC4, and a dilution of 1:200 is chosen for conjugate EC5.

6.17.3. <u>Inhibition of Labeled Conjugates by Affinity Receptor</u>

Commercially obtained mouse anti-HBsAg is diluted 2500,

1250, 625, 312, 160, 80 and 40 μg/mL in a buffer of 0.013 M Tris (pH 6.0), containing 1 g/L BSA, 20 g/L NaCl, and 1 mL/L Tween 20. A portion (100 μL) of each dilution is added to wells of a microtiter plate. Next, 25 μL of a solution of either conjugate EC4 (1:150 diluted solution) or EC5 (1:200 diluted solution) are added to each well, and the resulting mixtures allowed to incubate for 1 hour at room temperature. Control wells using solutions of non-immune mouse IgG are also prepared for each conjugate.

The mixtures are then warmed to 37 °C and 25 μL of 0.013 M Tris (pH 6.0), containing 1 g/L BSA, 20 g/L NaCl, 1 mL/L Tween 20, and 16.62 g/L NAD prewarmed to 37 °C are added to each of the wells. The mixtures are shaken for 1 minute, then G6PDH activity is monitored by measuring the absorbance at 340 nm every 30 seconds for 3 minutes at 37 °C.

The results, presented in the Table, below, idicate that approximately 25% inhibition can be observed with both conjugates at the indicated concentration of antibody. The labeled conjugates are subsequently used in an EMIT assay for the detection of hepatitis surface antigen in a sample.

TABLE 5

INHIBITION OF G6PDH-PEPTIDE CONJUGATE ACTIVITY WITH INCREASING MOUSE ANTI-HBsAg/NON-IMMUNE IgG

	RATES: mA/min at 340 nm											
	EC4*; 1:150						EC5*; 1:200					
	MOUSE ANTI-HBsAg			IgG NON-IMMUNE			MOUSE ANTI-HBsAg			IgG NON-IMMUNE		
CONC Ab (ug/mL)	1	2	MEAN	1	2	MEAN	1	2	MEAN	1	2	MEAN
0	198	203	200	184	210	197	254	282	268	264	267	265
40	205	194	199	181	200	190	251	278	264	269	256	263
80	200	196	198	186	194	190	255	283	269	259	271	266
160	203	206	204	212	202	207	251	265	258	267	270	268
312	193	191	192	181	197	189	252	257	255	272	252	262
625	180	189	184	196	194	195	248	254	251	271	246	258
1250	166	171	168	178	196	187	220	239	229	266	249	257
2500	147	152	149	200	191	196	191	207	199	274	253	263

* The labeled conjugate and antibody were pre-incubated for 1 hour at room temperature.

6.18. Method for Detection of Analyte of Interest With Labeled Mimetope

The following Example is for a method of determining the presence or absence of an hepatitis analyte in a sample by an antibody-mediated fluorescence enhancement affinity assay, such as one described by Wei, A-P, et al., in *Anal. Chem.* (1994) 66:1500-1506.

Hepatitis mimetope peptide of SEQ. ID. NO. 32 is allowed to react with tetramethylrhodamine-5-maleimide in 50 mM of phosphate buffer (pH 6) for 48 hours at 4 °C to make the labeled peptide. The

peptide is then reacted with 5-carboxyl-fluorescein succinimidyl ester in 50 mM borate buffer (pH 8.5) to double label the hepatitis peptide. FAB mass spectrometry is used to confirm the chemical identity of the doubly labeled peptide.

The doubly labeled peptide is combined with a sample suspected of containing the hepatitis analyte, along with an appropriate amount of anti-hepatitis antibody.

The fluorescence activity of the sample is measured upon excitation with an ISS PC-1 fluorometer (ISS, Champaign, IL).

The measured fluorescence enhancement is compared with the measured activity from a standard curve or the fluorescence observed from a control to determine the presence or absence of the hepatitis analyte in the sample.

6.19. Method for Detection of Analyte of Interest with Labeled Mimetope with Binding Profile for Antibody

The following Example is for a method of determining the presence or absence of an hepatitis antibody in a sample by an affinity assay.

Hepatitis mimetope peptide SEQ. ID. NO. 32, 35 or 37 is labeled with glucose-6-phosphate dehydrogenase as described above. FAB mass spectrometry is used to confirm the chemical identity of the labeled peptide. The labeled peptide is then used in an EMIT assay as described above to determine the presence or absence of hepatitis analyte in a given sample.

The observed rates are compared with the measured activity from a control to determine the presence or absence of the antibody in the sample. Alternatively, the amount or concentration of analyte

can be determined quantitatively with the appropriate measurements and controls.

6.20. Kit in Accordance with the Invention

Homogeneous immunoassay kit for performance of homogeneous assay of Example 18 comprises:

(a) a first container of functional surrogate, SEQ. ID. NO. 32, labeled with tetramethylrhodamine-5-maleimide and 5-carboxyl-fluorescein succinimidyl ester. The labeled peptide is capable of exhibiting an activity that is altered on binding of the labeled conjugate to the hepatitis affinity receptor and the activity can be measured and related to the amount of the analyte present in a given sample.

(b) a second container comprising goat or mouse anti-hepatitis surface antigen antibody.

6.21. EMIT Kit

For an EMIT assay, a kit is provided including a first container of a functional surrogate (e.g., SEQ. ID. NOS. 35 or 37) labeled with G6PDH. A second container is also provided containing an antibody against the particular analyte of interest and, optionally, the G6P and NAD substrates for the GSPDH. If desired, these substrates can be present in a separate container.

6.22. Recombinant DNA Construct Comprising a DNA Sequence Encoding a Functional Surrogate

A recombinant DNA construct is prepared which includes a DNA sequence encoding a hepatitis epitope or mimetope as obtained

by the methods of the invention This functional surrogate is capable of competing effectively for anti-hepatitis antigen antibody in the presence of the antigen. The recombinant DNA construct is made in accordance with methods known in the art such as those set forth in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989) using DNA inserts comprising the sequences set forth in this disclosure.

A transforming vector, including the above DNA construct, a bacteriophage transformed by the vector, and a microorganism such as *E. coli* or yeast transformed by the vector or infected with the bacteriophage are made by methods known in the art such as those set forth in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989) or other references available to the skilled artisan, such as some of the patents mentioned herein. Preferably, such transforming vectors will include an origin of replication functional in the host to allow for autonomous replication of the vector. Alternatively, the vector may integrate into the host chromosome.

In sum, the above disclosure teaches how to obtain, make and use functional surrogates having many uses, including, especially, use in homogenous enzyme immunoassays. The assay of the invention can advantageously detect macromolecular analytes, which have previously been difficult to assay for a number of reasons, as discussed above.

REFERENCES

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The purpose of the above description and examples is to illustrate some embodiments of the present invention without implying any limitation. It will be apparent to those of skill in the art that various modifications and variations may be made to the composition and method of the present invention without departing from the spirit or scope of the invention. All patents and publications cited herein are incorporated by reference in their entirety.

TABLE 6

MONOCLONAL ANTI-HBsAg BINDER SEQUENCES
FROM R26 LIBRARY

RELEVANT AMINO ACID SEQUENCES		SEQ. ID. NO.
FC23	MSMRSTVNVERRPAVAEPPAHLRINWGSR	176
FC41	VPTYWPSASILRSAETNGLHKLSHPLYSR	177
B26	ISSGLPSRLGCVSADAQTCHYHPIYNRSR	178
FC22	ACEIDPFYHPIYSAADQGARSDECIFPSR	179
FC21	DGSWWDMDLCSLPADCDALRSREKSRISR	180
FC32	LPGPPHLSVRHIPAESQNPTVDEAPAHSR	181
A28	TESAQRASSSTAASTHAVYGPPPNLSR	182
B13	ICAGASAGHQCRPAGPRHLDPSHSNGQSR	183
C10	VQSVSSVGLMPYAAVSVHNNVSDHPLYSR	184
C24	VSAGTPTHTASLAAVNNYRHHPIYNPTSR	185
D20	FRPMQESLKAVDAAAAPPPYQFPMDDQSR	186
D7	HDLWCTGPRHLCPADMFPGTSNPSPPSSR	187
FC11	DAMSGGTGTSLDAAVIGPGHLFEYVDVSR	188
FC15	NFHAPFNHGEVETAASYLTDVPPHLLWSR	189
FC16	MAYFSSIGPVEHPAAGPGPLPRDFPPSSR	190

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TABLE 6 (con'd.)

MONOCLONAL ANTI-HBsAg BINDER SEQUENCES FROM R26 LIBRARY

SEQUENCES ALIGNED BY APPARENT MOTIFS				SEQ. ID. NO.
C10	VQSVSSVGLMPYAAVSVHNNVSD	**HPLY**	SR	184
FC41	VPTYWPSASILRSAETNGLHKLS	**HPLY**	SR	177
B26	ISSGLPSRLGCVSADAQTCHY	**HPIY**	NRSR	178
FC22	ACEIDPFY	**HPIY**	SAADQGARSDECIFPSR	179
C24	VSAGTPTHTASLAAVNNYRH	**HPIY**	NPTSR	185
FC32	LP	**GPPHL**	SVRHIPAESQNPTVDEAPAHSR	181
FC16	MAYFSSIGPVEHPAA	**GPGPL**	PRDFPPSSR	190
FC11	DAMSGGTGTSLDAAVI	**GPGHL**	FEYVDVSR	188
D7	HDLWCT	**GPRHL**	CPADMFPGTSNPSPPSSR	187
B13	ICAGASAGHQCRPA	**GPRHL**	DPSHSNGQSR	183
FC15	NFHAPFNHGEVETAASYLTD	**VPPHL**	LWSR	189
FC23	MSMRSTVNVERRPAVAE	**PPAHL**	RINWGSR	176
A28	TESAQRASSSTAASTHAVYG	**PPPNL**	SR	182
FC22	ACEIDPFYHPIYSAADQG	**ARSDE**	CIFPSR	179
FC21	DGSWWDMDLCSLPADCDA	**LRSRE**	KSRISR	180
FC41	VPTYWPSASI	**LRSAE**	TNGLHKLSHPLYSR	177
D20	FRPMQESLKAVDAAAAPPPYQFPMDDQSR	-	no apparent motif	198

TABLE 6 (con'd)

MONOCLONAL ANTI-HBsAg BINDER SEQUENCES
FROM R26 LIBRARY

NUCLEOTIDE SEQUENCES SEQ. ID. NO.

FC.23 191

CAC	TCC	TCG	AGG	CTC	CCC	GGG	CCC	CCC
H	S	S	R	L	P	G	P	P
CAT	CTG	TCT	GTC	CGG	CAT	ATT	CCC	GCG
H	L	S	V	R	H	I	P	A
GAG	AGT	CAG	AAC	CCC	ACT	GTT	GAC	GAG
E	S	Q	N	P	T	V	D	E
GCT	CCC	GCT	CAT	TCT	AGA			
A	P	A	H	S	R			

FC.41 192

CAC	TCC	TCG	AGT	GTC	CCT	ACT	TAT	TGG
H	S	S	S	V	P	T	Y	W
CCT	AGC	GCT	TCT	ATC	CTC	AGA	TCC	GCG
P	S	A	S	I	L	R	S	A
GAG	ACC	AAC	GGG	TTG	CAC	AAG	CTT	GAC
E	T	N	G	L	H	K	L	D
CAC	CCC	CTT	TAT	TCT	AGA			
H	P	L	Y	S	R			

B.26 193

CAC	TCC	TCG	AGG	ATT	TCT	TCT	GGT	TTG
H	S	S	R	I	S	S	G	L
CCT	TCG	AGG	CTG	GGT	TGC	GTG	TCC	GCG
P	S	R	L	G	C	V	S	A
GAC	GCG	CAG	ACC	TGC	CAT	TAC	CAC	CCT
D	A	Q	T	C	H	Y	H	P
ATC	TAT	AAC	AGG	TCT	AGA			
I	Y	N	R	S	R			

FC.22 194

CAC	TCC	TCG	AGG	GCT	TGC	GAG	ATC	GAT
H	S	S	R	A	C	E	I	D
CCT	TTT	TAT	CAC	CCT	ATC	TAC	TCC	GCG
P	F	Y	H	P	I	Y	S	A
GCT	GAC	CAG	GGG	GCT	CGC	AGT	GAC	GAG
A	D	Q	G	A	R	S	D	E
TGT	ATT	TTC	CCG	TCT	AGA			
C	I	F	P	S	R			

TABLE 6 (con'd)

MONOCLONAL ANTI-HBsAg BINDER SEQUENCES
FROM R26 LIBRARY

NUCLEOTIDE SEQUENCES

SEQ. ID. NO.

FC.21 — SEQ. ID. NO. 195

CAC	TCC	TCG	AGC	GAT	GGG	AGT	TGG	TGG
H	S	S	S	D	G	S	W	W
GAT	ATG	GAT	CTC	TGT	TCG	CTG	CCC	GCG
D	M	D	L	C	S	L	P	A
GAC	TGT	GAT	GCC	TTG	CGC	TCG	CGC	GAG
D	C	D	A	L	R	S	R	E
AAG	AGC	CGG	ATC	TCT	AGA			
K	S	R	I	S	R			

FC.32 — SEQ. ID. NO. 196

CAC	TCC	TCG	AGG	CTC	CCC	GGG	CCC	CCC
H	S	S	R	L	P	G	P	P
CAT	CTG	TCT	GTC	CGG	CAT	ATT	CCC	GCG
H	L	S	V	R	H	I	P	A
GAG	AGT	CAG	AAC	CCC	ACT	GTT	GAC	GAG
E	S	Q	N	P	T	V	D	E
GCT	CCC	GCT	CAT	TCT	AGA			
A	P	A	H	S	R			

A.28 — SEQ. ID. NO. 197

CAC	TCC	TCG	ACA	ACA	GAG	TCT	GCG	CAG
H	S	S	T	T	E	S	A	Q
AGA	GCC	TCT	TCA	TCA	ACC	GCG	GCC	TCC
R	A	S	S	S	T	A	A	S
ACC	CAC	GCC	GTC	TAC	GGC	CCT	CCC	CCT
T	H	A	V	Y	G	P	P	P
AAT	CTT	TCT	AGA					
N	L	S	R					

B.13 — SEQ. ID. NO. 198

CAC	TCC	TCG	AGC	ATT	TGC	GCT	GGT	GCT
H	S	S	S	I	C	A	G	A
TCT	GCT	GGC	CAC	CAG	TGC	CGT	CCC	GCG
S	A	G	H	Q	C	R	P	A
GGT	CCC	GCG	CAC	TTG	GAT	CCG	AGT	CAC
G	P	R	H	L	C	P	S	H
TCG	AAC	GGC	CAG	TCT	AGA			
S	N	G	Q	S	R			

TABLE 6 (con'd)

MONOCLONAL ANTI-HBsAg BINDER SEQUENCES
FROM R26 LIBRARY

<u>NUCLEOTIDE SEQUENCES</u> <u>SEQ. ID. NO.</u>

C.10 199

CAC	TCC	TCG	AGC	GTT	CAG	TCT	GTG	AGC
H	S	S	S	V	Q	S	V	S
AGC	GTT	GGG	TTG	ATG	CCT	TAC	GCC	GCG
S	V	G	L	M	P	Y	A	A
GTG	AGC	GTT	CAC	AAC	AAT	GTC	TCT	GAC
V	S	V	H	N	N	V	S	D
CAT	CCG	CTC	TAT	TCT	AGA			
H	P	L	Y	S	R			

C.24 200

CAC	TCC	TCG	AGC	GTG	AGT	GCG	GGT	ACC
H	S	S	S	V	S	A	G	T
CCG	ACC	CAC	ACG	GCG	AGC	TTG	GCC	GCG
P	T	H	T	A	S	L	A	A
GTG	AAT	AAC	TAT	CGT	CAC	CAT	CCC	ATT
V	N	N	Y	R	H	H	P	I
TAT	AAC	CCG	ACT	TCT	AGA			
Y	N	P	T	S	R			

D.20 201

CAC	TCC	TCG	AGC	TTT	CGC	CCG	ATG	CAG
H	S	S	S	F	R	P	M	Q
GAG	AGT	CTT	AAG	GCC	GTC	GAC	GCC	GCG
E	S	L	K	A	V	D	A	A
GCT	GCG	CCC	CCC	CCC	TAC	CAG	TTC	CCT
A	A	P	P	P	Y	Q	F	P
ATG	GAC	GAT	CAG	TCT	AGA			
M	D	D	Q	S	R			

D.7 202

CAC	TCC	TCG	AGT	CAC	GAC	TTG	TGG	TGT
H	S	S	S	H	D	L	W	C
ACT	GGT	CCG	CGC	CAT	TTG	TGC	CCC	GCG
T	G	P	R	H	L	C	P	A
GAT	ATG	TTC	CCA	GGC	ACG	AGC	AAC	CCC
D	M	F	P	G	T	S	N	P
AGC	CCG	CCT	AGC	TCT	AGA			
S	P	P	S	S	R			

TABLE 6 (con'd)

MONOCLONAL ANTI-HBsAg BINDER SEQUENCES
FROM R26 LIBRARY

NUCLEOTIDE SEQUENCES

SEQ. ID. NO.

FC.11

CAC H	TCC S	TCG S	AGC S	GAC D	GCC A	ATG M	TCG S	GGT G	203
GGT G	ACG T	GGT G	ACG T	TCC S	CTA L	GAT D	GCC A	GCG A	
GTT V	ATT I	GGT G	CCG P	GGC G	CAC H	CTT L	TTT F	GAG E	
TAT Y	GTC V	GAC D	GTC V	TCT S	AGA R				

FC.15

CAC H	TCC S	TCG S	AGC S	AAT N	TTT F	CAC H	GCC A	CCT P	204
TTC F	AAC N	CAC H	GGT G	GAG E	GTC V	GAG E	ACC T	GCG A	
GCC A	TCG S	TAC Y	TTG L	ACC T	GAT D	GTC V	CCC P	CCC P	
CAT H	CTG L	CTC L	TGG W	TCT S	AGA R				

FC.16

CAC H	TCC S	TCG S	AGC S	ATG M	GCC A	TAC Y	TTT F	TCC S	205
TCC S	ATT I	GGT G	CCC P	GTG V	GAG E	CAT H	CCC P	GCG A	
GCT A	GGC G	CCC P	GGG G	CCC P	CTT L	CCC P	CGT R	GAT D	
TTT F	CCT P	CCG P	TCC S	TCT S	AGA R				

TABLE 7

MONOCLONAL ANTI-HBsAg BINDER SEQUENCES
FROM RBC LIBRARY

RELEVANT AMINO ACID SEQUENCES		SEQ ID NO:
M10	CGGPEHLQVC	
M13	CARGEVLPKC	206
M20	CSGPKHLQVC	207
M3	CGGRGASSRC	208
M4	CQWWGGRDKC	209
M8	CDWKTVLPRC	210
M12	CSNGGPDHLC	211
M18	CDGPRHLSTC	212
M23	CEEGAVLPRC	213
M24	CKCHPLYGGC	214
M29	CEQGAVLAKC	215
		216

SEQUENCES ALIGNED BY APPARENT MOTIFS				SEQ ID NO:
M24	CKC	HPLY	GGC	
M8	CDW	KTVLPR	C	215
M13*	CAR	GEVLPK	C	211
M23	CEE	GAVLPR	C	207
M29	CEQ	GAVLAK	C	214
				216
M20*	CS	GPKHL	QVC	
M12	CSNG	GPDHL	C	208
M18	CD	GPRHL	STC	212
M10*	CG	GPEHL	QVC	213
				206
M3	CGGRGASSRC	No apparent motif		
M4	CQWWGGRDKC	No apparent motif		209
				210

TABLE 7 (con'd)

NUCLEOTIDE SEQUENCES

SEQ ID. NO.

M.10

CAC L CTT	TCC Q CAG	TCG V GTC	AGT C TGT	C TGT G GGA	G GGG S TCT	G GGG R AGA	P CCG	E GAG	H CAT	217

M.13

CAC P CCT	TCC K AAG	TCG C TGT	AGT G GGA	C TGT S TCT	A GCG R AGA	R AGG	G GGG	E GAG	V GTG	218 L TTG

M.20

CAC Q CAG	TCC V GTG	TCG C TGT	AGT G GGA	C TGT S TCT	S AGT R AGA	G GGG	P CCT	K AAG	H CAT	219 L TTG

M.3

CAC K AAG	TCC V GTC	TCG C TGT	AGT GGA	C TGT TCT	G GGG AGA	G GGC	R CGG	S AGC	I ATC	220 F TTC

M.4

CAC D GAT	TCC K AAG	TCG C TGT	AGT G GGA	C TGT S TCT	Q CAA R AGA	W TGG	W TGG	G GGG	G GGG	221 R CGG

M.8

CAC L CTG	TCC P CCG	TCG R AGG	AGT C TGT	C TGT G GGA	D GAT S TCT	W TGG R AGA	K AAG	T ACG	V GTT	222

TABLE 7 (con'd)

NUCLEOTIDE SEQUENCES												SEQ. ID NO.
M.12	CAC H CAT	TCC L CTC	TCG C TGT	AGT G GGA	C TGT S TCT	S TCT R AGA	N AAT	G GGG	G GGT	P CCG	D GAT	223
M.18	CAC S TCT	TCC T ACG	TCG C TGT	AGT G GGA	C TGT S TCT	D GAT R AGA	G GGG	P CCT	R CGT	H CAT	L TTG	224
M.23	CAC L TTG	TCC P CCG	TCG R CGG	AGT C TGT	C TGT G GGA	E GAG S TCT	E GAG R AGA	G GGT	A GCG	V GTG		225
M.24	CAC Y TAT	TCC G GGG	TCG G GGT	AGT C TGT	C TGT G GGA	K AAG S TCT	C TGT R AGA	H CAT	P CCT	L CTG		226
M.29	CAC L TTG	TCC A GCG	TCG K AAG	AGT C TGT	C TGT G GGA	E GAG S TCT	Q CAG R AGA	G GGT	A GCG	V GTT		227

TABLE 8

POLYCLONAL ANTI-HBSAg BINDER SEQUENCES
FROM R26 LIBRARY

RELEVANT AMINO ACID SEQUENCES

A3		
D8	STSSIGPLRHHAMTADSPHTGIDFHGGP	SEQ ID NO:
C2	SADSNTPRGPLKYSADRLYTPDGLGMQA	
A12	IVWVAEQGESTTSAGPRKAIPDLSDSR	228
D10	LDFRCPSDGNCYAAAPLEPQLGVRNSL	229
C14	RCPSDGLCYFGVDRGHWRSPHHPSAPT	230
B18	STTTIXKTKDREVTADPSATSSIQNIGR	231
B13	DGFSSAFFSGTARGPTKLGVLPSPQA	232
	SLTSSGPIKGALAADSQSKPYSGPIMP	233
		234
		235

SEQUENCES ALIGNED BY APPARENT MOTIFS

A3				
D8	SAD	STSSIGPLR	HHAMTADSPHTGIDFHGGP	SEQ ID NO:
C2	IVWVAEQGE	SNTPRGPLK	YSADRLYTPDGLGMQA	228
C14		STTSAGPRK	AIPDLSDSR	229
B18'	XGFSSAFF	STTTIXKTK	DREVTADPSATSSIQNIGR	230
B13'		SGTARGPTK	LGVLPSPQX	233
		SLTSSGPIK	GALAADSQSKPYSGPIMP	234
A12	LDF			235
D10		RCPSDGNCY	AAAPLEPQLGVRNSL231	
		RCPSDGLCY	FGVDRGHWRSPHHPSAPT	
				232

TABLE 8 (con'd)

SELECTED NUCLEOTIDE SEQUENCES	SEQ. ID. NO
B18	
CAC A GCT P CCT P CCG TCC F TTC T ACC Q CAG TCG F TTT K AAG A GCC AGT S AGT L TTG S TCT D GAC G GGT G GGC R AGA G GGG T ACG V GTG F TTC A GCC L CTT S AGC R CGC P CCC S AGC G GGG S AGC	236
B13	
CAC P CCG Q CAG P CCT TCC I ATT S TCC S TCT TCG K AAG K AAG R AGA AGT G GGG P CCT S TCT A GCG Y TAC L CTT L TTG S TCT T ACG A GCC G GGT S TCT A GCG P CCT S AGT D GAT I ATT G GGC S TCT M ATG	237

SUBSTITUTE SHEET (RULE 26)

TABLE 9

POLYCLONAL ANTI-HBsAg BINDER SEQUENCES
FROM R8C LIBRARY

RELEVANT AMINO ACID SEQUENCES		SEQ. ID. NO.
P7	CWLNWRGGTC	238
P8	CRGGDRHPGC	239
P10	CWEPYRGANC	240
P19	CGQICRQSLC	241

No apparent motifs from the above

TABLE 10

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (A SERIES)

RELEVANT AMINO ACID SEQUENCES		SEQ ID NO:
A1	HSSSSQHGGSAMFSLSSAAHSPAAHQATHTSSR	242
A3	HSSSFQLGSGGEALFKSAAALGPPGSRTPFHSR	243
A4	HSSSSASPTSVTFLRQPAVSGGRSLFQNLDPSR	244
A6	HSSRDLFHGGQAMFNSAAVAAKSSGLISPDSSR	245
A7	HSSSKYGGMSLFQSQMTAGHHAGTPPYTSRWSR	246
A8	HSSSSALFQSVAPLFSSAAPSNNDRSPKPFTSR	247
A9	HSSSLAYSPIGASLFQSAANNPSIPRRTSDVSR	248
A11	HSSSLQLFTTALPWRDTAAPPMLSNSALFQMSR	249
A12	HSSSAAGGTSENQNSWAAVAGGASLFQNSHRSR	250
A13	HSSSFRSSPHGRAMFQSAGNGSFGNVPALSSSR	251
A14	HSSSTRTSQVSYGVSRPAAASHSPQRAFFQVSR	252
A21	HSSSPWNVNAKNDDGMAAGRALFKQALNNGTSR	253
A24	HSSRAFVPTFPMMTIRSAGRALFHECRNDHASR	254
A27	HSSSTVSFKRPGFEQMAAGLQQGQSSINPTPSR	255

SEQUENCES ALIGNED BY APPARENT MOTIFS				SEQ ID NO:
A24	HSSRAFVPTFPMMTIRS	**AGRALFH**	ECRNDHASR	254
A13	HSSSFRSSP	**HGRAMFQ**	SAGNGSFGNVPALSSSR	251
A6	HSSRDLFH	**GGQAMFN**	SAAVAAKSSGLISPDSSR	244
A1	HSSSSQH	**GGSAMFS**	LSSAAHSPAAHQATHTSSR	241
A3	HSSSFQLGS	**GGEALFK**	SAAALGPPGSRTPFHSR	243
A4	HSSSSASPTSVTFLRQPAVS	**GGRSLFQ**	NLDPSR	244
A7	HSSSKY	**GGMSLFQ**	SQMTAGHHAGTPPYTSRWSR	246
A12	HSSSAAGGTSENQNSWAAVA	**GGASLFQ**	NSHRSR	250
A21	HSSSPWNVNAKNDDGMA	**AGRALFK**	QALNNGTSR	253
A9	HSSSLAYSP	**IGASLFQ**	SAANNPSIPRRTSDVSR	248
A8	HS	**SSSALFQ**	VAPLFSSAAPSNNDRSPKPFTSR	247
A11	HSSSLQLFTTALPWRDTAAPPML	**SNSALFQ**	MSR	249
A14	HSSSTRTSQVSYGVSRPAAASHS	**PQRAFFQ**	VSR	252
A27	HSSSTVSFKRPGFEQMAAGLQQGQS	**SINPTP**	SR	255

TABLE 10 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (A SERIES)

NUCLEOTIDE SEQUENCES

A.1

									SEQ ID NO:
				S	Q	H	G	G	256
CAC	TCC	TCG	AGT	TCT	CAG	CAC	GGG	GGG	
S	A	M	F	S	L	F	S	A	
AGC	GCC	ATG	TTT	AGC	CTG	TTC	TCC	GCG	
A	H	S	P	A	A	N	Q	A	
GCT	CAT	AGC	CCC	GCT	GCT	CAT	CAG	GCG	
T	H	T	S	S	R				
ACG	CAC	ACC	AGC	TCT	AGA				

A.3

									SEQ ID NO:
				F	Q	L	G	S	257
CAC	TCC	TCG	AGT	TTT	CAG	TTG	GGC	TCC	
G	G	E	A	L	F	K	S	A	
GGC	GGG	GAG	GCG	CTT	TTT	AAG	TCC	GCG	
A	A	L	G	P	P	G	S	R	
GCC	GCG	CTC	GGC	CCC	CCG	GGG	TCC	CGC	
T	P	F	H	S	R				
ACG	CCG	TTT	CAC	TCT	AGA				

A.4

									SEQ ID NO:
				S	A	S	P	T	258
CAC	TCC	TCG	AGC	AGC	GCG	AGC	CCC	ACC	
S	V	T	F	L	R	Q	P	A	
AGC	GTC	ACT	TTT	TTG	CGG	CAG	CCC	GCG	
V	S	G	G	R	S	L	F	Q	
GTG	AGT	GGG	GGG	CGT	AGT	CTC	TTC	CAG	
N	L	D	P	S	R				
AAC	CTC	GAT	CCC	TCT	AGA				

A.6

									SEQ ID NO:
			R	D	L	F	H	G	259
CAC	TCC	TCG	AGG	GAT	CTT	TTC	CAT	GGG	
G	Q	A	M	F	N	S	A	A	
GGT	CAG	GCT	ATG	TTT	AAC	TCG	GCC	GCG	
V	A	A	K	S	S	G	L	I	
GTG	GCT	GCT	AAG	AGC	TCG	GGT	TTG	ATC	
S	P	D	S	S	R				
AGT	CCC	GAC	TCC	TCT	AGA				

TABLE 10 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (A SERIES)

NUCLEOTIDE SEQUENCES

SEQ. ID. NO.

A.7

			S	K	Y	G	G	M	260
CAC	TCC	TCG	AGC	AAG	TAT	GGT	GGT	ATG	
S	L	F	Q	S	Q	M	T	A	
AGT	TTG	TTT	CAG	TCG	CAG	ATG	ACC	GCG	
G	H	H	A	G	T	P	P	Y	
GGC	CAT	CAT	GCG	GGG	ACC	CCC	CCG	TAT	
T	S	R	W	S	R				
ACG	TCC	AGG	TGG	TCT	AGA				

A.8

			S	S	A	L	F	Q	261
CAC	TCC	TCG	AGC	AGT	GCG	TTG	TTT	CAG	
S	V	A	P	L	F	S	S	A	
TCG	GTC	GCC	CCC	CTG	TTT	TCG	TCC	GCG	
A	P	S	N	N	D	R	S	P	
GCG	CCG	TCG	AAC	AAT	GAC	CGG	TCG	CCC	
K	P	F	T	S	R				
AAG	CCC	TTC	ACT	TCT	AGA				

A.9

			S	L	A	Y	S	P	262
CAC	TCC	TCG	AGT	TTG	GCG	TAC	TCT	CCC	
I	G	A	S	L	F	Q	S	A	
ATC	GGT	GCT	TCG	TTG	TTT	CAG	TCC	GCG	
A	N	N	P	S	I	P	R	R	
GCT	AAC	AAC	CCG	AGC	ATC	CCC	CGT	CGT	
T	S	D	V	S	R				
ACT	TCC	GAT	GTT	TCT	AGA				

A.11

				L	Q	L	F	T	263
CAC	TCC	TCG	AGC	TTG	CAG	CTG	TTT	ACC	
T	A	L	P	W	R	D	T	A	
ACT	GCT	TTG	CCG	TGG	AGG	GAC	ACC	GCG	
A	P	P	M	L	S	N	S	A	
GCG	CCG	CCG	ATG	CTT	TCC	AAC	AGC	GCC	
L	F	Q	M	S	R				
CTT	TTT	CAG	ATG	TCT	AGA				

TABLE 10 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (A SERIES)

NUCLEOTIDE SEQUENCES SEQ ID NO:

A.12 264

				A	A	G	G	T
CAC	TCC	TCG	AGC	GCT	GCC	GGG	GGG	ACC
S	E	N	Q	N	S	W	A	A
TCG	GAG	AAT	CAG	AAC	AGT	TGG	GCC	GCG
V	A	G	G	A	S	L	F	Q
GTT	GCC	GGG	GGC	GCG	AGT	CTT	TTT	CAG
N	S	H	R	S	R			
AAT	AGC	CAC	CGG	TCT	AGA			

A.13 265

				F	R	S	S	P
CAC	TCC	TCG	AGT	TTT	CGG	TCC	TCG	CCC
H	G	R	A	M	F	Q	S	A
CAC	GGC	CGG	GCT	ATG	TTC	CAG	TCC	GCG
G	N	G	S	F	G	N	V	P
GGC	AAC	GGG	AGT	TTT	GGG	AAT	GTC	CCG
A	L	S	S	S	R			
GCT	CTG	TCC	AGC	TCT	AGA			

A.14 266

				T	R	T	S	Q
CAC	TCC	TCG	AGT	ACG	CGC	ACT	TCC	CAG
V	S	Y	G	V	S	R	P	A
GTC	TCG	TAT	GGG	GTC	AGT	CGT	CCC	GCG
A	A	S	H	S	P	Q	R	A
GCC	GCT	TCG	CAT	TCG	CCT	CAG	AGG	GCT
F	F	Q	V	S	R			
TTC	TTC	CAG	GTC	TCT	AGA			

A.21 267

CAC	TCC	TCG	AGC	CCT	TGG	AAT	GTG	AAT
H	S	S	S	P	W	N	V	N
GCC	AAG	AAC	GAC	GAC	GGT	ATG	GCC	GCG
A	K	N	D	D	G	M	A	A
GGG	CGT	GCC	CTT	TTT	AAG	CAG	GCG	CTC
G	R	A	L	F	K	Q	A	L
AAT	AAC	GGG	ACT	TCT	AGA			
N	N	G	T	S	R			

TABLE 10 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (A SERIES)

<u>NUCLEOTIDE SEQUENCES</u>

<u>SEQ ID NO:</u>

A.24

				M	F	Q	E	H	268
CAC	TCC	TCG	AGT	ATG	TTT	TAG	GAG	CAT	
R	T	N	L	Q	L	D	T	A	
AGG	ACT	AAT	TTG	CAG	AAG	GAC	ACC	GCG	
D	R	S	F	T	P	G	Y	R	
GAC	CGT	TCC	TTT	ACC	CCT	GGT	TAT	CGC	
T	D	L	D	S	R				
ACG	GAT	TTG	GAT	TCT	AGA				

A.27

				T	V	S	F	K	269
CAC	TCC	TCG	AGT	ACG	GTT	AGC	TTT	AAG	
R	P	G	F	E	Q	M	A	A	
CGG	CCC	GGG	TTT	GAG	CAG	ATG	GCC	GCG	
G	L	Q	Q	G	Q	S	S	I	
GGT	CTG	CAG	CAG	GGA	CAG	AGT	TCC	ATC	
N	P	T	P	S	R				
AAC	CCC	ACC	CCC	TCT	AGA				

TABLE 11

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (B SERIES)

RELEVANT AMINO ACID SEQUENCES		SEQ. ID. NO.
B1	HS LELSINGTPVMGYTPRNIQEPKLSTDRNAR	270
B10	HSSSFSITSMGWSGATSAVSGGSSFWQHYVHSR	271
B11	HSSSCYFCDTGVGAPASAGTWSANGNNIHLTSR	272
B13	HSSSKDSFFQIDRLRSTAVNRIASNHPPMPNSR	273
B17	HSSSIDGIQGHSGLFGTAASRGIGNTVMFQASR	274
B18	HSSSGGYKLHAGERNLAAAYAGTSSGERGLTSR	275
B20	HSSRQITAHPLTSVANLRGGDALFTQMRLHHSR	276
B22	HSSSLGNYNRGGMALFTAASSSRGQATERPVSR	277
B23	HSSSSMFCGAMFCQSSSAEHSRTTFKEANYLSR	278
B3	HSSSIVKQSVDVNLQVSADSPGTPASAFFQISR	279
B25	HSSSLFQENKLRGFLMSAGPST NRASTIDGSR	280
B27	HSSSASNGSSLFNDLKPAGGKLKLAPRATGISR	281

SEQENCES ALIGNED BY APPARENT MOTIFS

				SEQ ID NO:
B1	HS LEL	SINGTP	VMGYTPRNIQEPKLSTDRNAR	270
B10	HSSSFSITSMGWSAGTSAVS	GGSSFWQ	HYVHSR	271
B13	HSS	SKDSFFQ	IDDRLRSTAVNRIASNHPPMPNSR	273
B17	HSSSIDGIQGHSGLFGTAASRGI	GNTVMFQ	ASR	274
B20	HSSRQITAHPLTSVANLR	GGDALFT	QMRLHHSR	276
B22	HSSSLGNYNR	GGMALFT	AASSSRGQATERPVSR	277
B23	HSSSSMF	CGAMFCQ	SSSAEHSRTTFKEANYLSR	278
B3	HSSSIVKQSVDVNLQVSADSPGT	PASAFFQ	ISR	279
B25		HSSSLFQ	INKLRGFLMSAGPSTXNARASTIDGSR	280
B27	HSSSAS	NGSSLFN	DLKPAGGKLKLAPRATGISR	281
B11	HSSSCYFCDTGVGAPASAGTWSANGNNIHLTSR			272
B18	HSSSGGYKLHAGERNLAAAYAGTSSGERGLTSR			275

TABLE 11 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (B SERIES)

NUCLEOTIDE SEQUENCES SEQ ID NO:

B.1 282

		L	E	L	S	I	N	G
CAC	TCC	CTA	GAA	CTC	AGC	ATA	AAC	GGA
T	P	V	M	G	Y	T	P	R
ACC	CCC	GTG	ATG	GGA	TAC	ACG	CCG	CGG
N	I	Q	E	P	K	L	S	T
AAC	ATC	CAG	GAG	CCC	AAA	CTC	AGC	ACA
D	R	N	A	R	P	S	R	
GAC	CGG	AAC	GCT	CGA	CCT	TCG	AGA	

B.10 283

				F	S	I	T	S
CAC	TCC	TCG	AGC	TTC	TCC	ATT	ACC	TCC
M	G	W	S	G	A	T	S	A
ATG	GGT	TGG	TCC	GGT	GCC	ACC	TCC	GCG
V	S	G	G	S	S	F	W	Q
GTG	AGC	GGT	GGT	TCG	AGC	TTC	TGG	CAG
H	Y	V	H	S	R			
CAT	TAT	GTG	CAC	TCT	AGA			

B.11 284

				C	Y	F	C	D
CAC	TCC	TCG	AGT	TGT	TAT	TTT	TGT	GAC
T	G	V	G	A	P	A	S	A
ACG	GGT	GTT	GGC	GCT	CCT	GCG	TCC	GCG
G	T	W	S	A	N	G	N	N
GGC	ACC	TGG	TCT	GCT	AAC	GGG	AAC	AAT
I	H	L	T	S	R			
ATC	CAC	TTH	ACG	TCT	AGA			

B.13 285

				K	D	S	F	F
CAC	TCC	TCG	AGC	AAG	GAT	TCG	TTC	TTT
Q	I	D	R	L	R	S	T	A
CAG	ATT	GAT	CGT	CTG	AGG	AGT	ACC	GCG
V	N	R	I	A	S	N	H	P
GTG	AAC	CGG	ATT	GCG	TCT	AAT	CAT	CCC
P	M	P	N	S	R			
CCC	ATG	CCG	AAT	TCT	AGA			

TABLE 11 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (B SERIES)

NUCLEOTIDE SEQUENCES

SEQ ID NO:

B.17 286

CAC G GGG T ACC T ACT
TCC H CAC A GCG V GTG
TCG S AGT A GCC M ATG
AGC G GGT S TCT F TTT
I ATC L TTG R AGG Q CAG
D GAT F TTT G GGT A GCC
G GGT G GGG I ATT S TCT
I ATC G GGG R AGA
Q CAG N AAC

B.18 287

CAC H 70 CAT H GCT A CGT E
TCC S GCC A TAT Y GGT R
TCG S GGT G GCC A 10 CTT G
AGC S GAG E GGT G ACT T
GGT G CGG R ACC T TCT S
GGG G AAT N AGT S AGA R
TAC Y TTG L TCC S
AAG K GCC A GGT G
TTG L GCG A GAG E

B.20 288

CAC P CCT G GGA R CGC
TCC L CTA G GGA L CTG
TCT T ACG D GAT H CAC
AGA S AGC A GCC H CAT
Q CAG V GTG L CTT S TCT
I ATT A GCT F TTC R AGA
T ACC N AAT T ACC
A GCA L CTC Q CAG
H CAC R CGC M ATG

B.22 289

CAC H CGT R GCT A GAG E
TCC S GGT G AGC S CGG R
TCG S GGG G TCG S CCC P
AGT S ATG M TCT S GTT V
TTG L GCG A CGG R TCT S
GGT G TTG L GGT G AGA R
AAT N TTT F CAG Q
TAT Y ACC T GCC A
AAT N GCG A ACG T

TABLE 11 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (B SERIES)

NUCLEOTIDE SEQUENCES SEQ ID NO:

B.23 290

				S	M	F	C	G
CAC	TCC	TCG	AGC	TCG	ATG	TTT	TGC	GGC
A	M	F	C	Q	S	S	S	A
GCG	ATG	TTC	TGT	CAG	AGC	TCT	TCC	GCG
E	H	S	R	T	T	F	K	E
GAG	CAC	TCC	CGT	ACC	ACG	TTT	AAG	GAG
A	N	Y	L	S	R			
GCT	AAT	TAC	CTG	TCT	AGA			

B.3 291

				I	V	K	Q	S
CAC	TCC	TCG	AGC	ATC	GTC	AAG	CAG	TCT
V	D	V	N	L	Q	V	S	A
GTT	GAT	GTT	AAT	TTG	CAG	GTC	TCC	GCG
D	S	P	G	T	P	A	S	A
GAC	AGC	CCT	GGG	ACG	CCG	GCT	AGC	GCC
F	F	Q	I	S	R			
TTT	TTC	CAG	ATT	TCT	AGA			

B.25 292

CAC	TCC	TCG	AGT	TTG	TTC	CAG	GAG	AAT
H	S	S	S	L	F	Q	E	N
70								
AAG	TTG	AGG	GGC	TTC	TTG	ATG	TCC	GCG
K	L	R	G	F	L	M	S	A
GGT	CCT	AGT	ACC	NAC	AAT	CGG	GCG	TCC
G	P	S	T		N	R	A	S
		10						
ACG	ATC	GGC	GAT	TCT	AGA			
T	I	G	D	S	R			

B.27 293

CAC	TCC	TCG	AGC	GCC	TCT	AAC	GGG	TCG
H	S	S	S	A	S	N	G	S
70								
TCG	CTT	TTT	AAT	GAC	TTG	AAG	CCC	GCG
S	L	F	N	D	L	K	P	A
GGT	GGG	AAG	CTT	AAG	CTG	GCC	CCG	CGC
G	G	K	L	K	L	A	P	R
				10				
GCC	ACG	GGT	ATT	TCT	AGA			
A	T	G	I	S	R			

TABLE 12

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (C SERIES)

RELEVANT AMINO ACID SEQUENCES

		SEQ ID NO:
C2	HSSSSSLQTGGRAFFLTAGNPGGSTAIPGGLSR	294
C10	HSSRPLWQVDA KAADTAEYYLSHD SHPSDSR	295
C12	HSSRSQSS FQLHSSTPAERMSTMRLNVPDASR	296
C14	HSSSLFQLRSSDKHPQAAGTSSASFGN SNHSR	297
C18	HSSSLFQIEAVPHWRRPADSHQLRAIHPHGDSR	298
C20	HSSSWRYSEVTAHDIPAAGAPLFQHERYLT SR	299
C21	HSSSPGSL KSITDRNSAAAPAPSSNPLPSRSR	300
C23	HSSSLFQNVVEGREMY AESPTTNILFHRHGSR	301
C26	HSPSGGQVMWRLSNLDSADR KTKAHASGVSSR	302
C28	HSSSTMLCLNPLCWTAAGRLDTYTNPSTTSR	303
C29	HSPRPK SELDSVNYWPAGRAFFRDFPT LASR	304

SEQUENCES ALIGNED BY APPARENT MOTIFS

				SEQ ID NO:
C2	HSSSSSLQT	GGRAFFL	TAGNPGGSTAIPGGLSR	294
C29	HSPRPK SELDSVNYWP	AGRAFFR	DFPT LASR	304
C12	HSSR	SQSS FQ	LHSSTPAERMSTMRLNVPDASR	296
C23		HSSSLFQ	NVVEGREMY AESPTTNILFHRHGSR	301
C14		HSSSLFQ	LRSSDKHPQAAGTSSASFGN SNHSR	297
C18		HSSSLFQ	IEAVPHWRRPADSHQLRAIHPHGDSR	298
C20	HSSSWRYSEVTAHDIPA	AGAPLFQ	HERYLT SR	299

C10	HSSRPLWQVDA KAADTAEYYLSHD SHPSDSR	- no apparent motifs	295
C21	HSSSPGSL KSITDRNSAAAPAPSSNPLPSRSR		300
C26	HSPSGGQVMWRLSNLDSADR KTKAHASGVSSR		302
C28	HSSSTMLCLNPLCWTAAGRLDTYTNPSTTSR		303

TABLE 12 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (C SERIES)

NUCLEOTIDE SEQUENCES

SEQ ID NO:

C.2 305

TCT	CAC	TCC	TCG	AGT	TCC	AGC	TTG	CAG
S	H	S	S	S	S	S	L	Q
ACG	GGT	GGC	CGG	GCG	TTC	TTT	TTG	ACC
T	G	G	R	A	F	F	L	T
GCG	GGT	ATT	CCG	GGC	GGT	TCC	ACT	GCC
A	G	N	P	G	G	S	T	A
ATC	CCG	GGT	GGC	CTC	TCT	AGA	CCN	TCG
I	P	G	G	L	S	R	P	S
AGA								
R								

C.10 306

TCT	CAC	TCC	TCG	AGG	CCT	CTG	TGG	CAG
S	H	S	S	R	P	L	W	Q
GTT	GAT	GCT	NTG	AAG	GCT	GCG	GAC	ACC
V	D	A		K	A	A	D	T
GCG	GAG	TAT	TAT	CTC	TCT	CAT	GAC	CGN
A	E	Y	Y	L	S	H	D	
TCG	CAC	CCG	TCG	GAC	TCT	AGA	CCA	TCG
S	H	P	S	D	S	R	P	S
AGA								
R								

C.12 307

TCT	CAC	TCC	TCG	AGG	AGT	CAG	AGT	TCT
S	H	S	S	R	S	Q	S	S
CNT	TTT	CAG	TTG	CAT	TCC	TCC	ACG	CCC
	F	Q	L	H	S	S	T	P
GCG	GAG	CGC	ATG	AGC	ACC	ATG	CGC	CTT
A	E	R	M	S	T	M	R	L
AAT	GTG	CCC	GAT	GCG	TCT	AGA	CCN	TCG
N	V	P	D	A	S	R	P	S
AGA								
R								

TABLE 12 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (C SERIES)

NUCLEOTIDE SEQUENCES SEQ ID NO:

C.14 308

TCT S	CAC H	TCC S	TCG S	AGT S	CTT L	TTC F	CAG Q	TTG I
AGG E	TCT A	AGT V	GAC P	AAG H	CAC W	CCG R	CAG R	GCC P
GCG A	GGT D	ACC S	TCG H	TCC Q	GCG L	AGC R	TTT A	GGC I
AAT H	NCC P	AGT H	AAT G	CAC D	TCT S	AGA R	CCA P	TCG S
AGA R								

C.18 309

TCT S	CAC H	TCC S	TCG S	AGT S	CTT L	TTT F	CAG Q	ATT I
GAG E	GCG A	GTG V	CCT P	CAC H	TGG W	AGG R	CGG R	CCC P
GCG A	GAC D	AGC S	CAT H	CAG Q	CTC L	CGG R	GCG A	ATT I
CAC H	CCC P	CAT H	GGG G	GAT D	TCT S	AGA R	CCN P	TCG S
AGA R								

C.20 310

TCT S	CAC H	TCC S	TCG S	AGT S	CTG L	TTT F	TAG U	AAT N
GTG V	GTC V	GAG E	GGT G	CGT R	GAG E	ATG M	TAT Y	NCC
GCG A	GAG E	AGC S	CCT P	ACC T	ACC T	AAC N	ATT I	CTT L
TTC F	CAT H	CGT R	CAT H	GGG G	TCT S	AGA R	CCN P	TCG S
AGA R								

TABLE 12 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (C SERIES)

NUCLEOTIDE SEQUENCES

SEQ ID NO:

C.21 311

TCT S	CAC H	TCC S	TCG S	AGT S	CCT P	GGG G	TCT S	CTT L
NCC	AAG K	AGT S	ATT I	ACT T	GAT D	AGG R	AAT N	TCC S
GCG A	GCT A	GCC A	CCC P	GCT A	CCG P	TCC S	TCC S	AAT N
CCT P	CTG L	CCT P	TCC S	AGG R	TCT S	AGA R	CCA P	TCG S
AGA R								

C.23 312

TCT S	CAC H	TCC S	TCG S	AGT S	CTG L	TTT F	TAG Q	AAT N
GTG V	GTC V	GAG E	GGT G	CGT R	GAG E	ATG M	TAT Y	NCC
GCG A	GAG E	AGC S	CCT P	ACC T	ACC T	AAC N	ATT I	CTT L
TTC F	CAT H	CGT R	CAT H	GGG G	TCT S	AGA R	CCN P	TCG S
AGA R								

C.26 313

TCT S	CAC H	TCC S	CCG P	AGT S	GGG G	GGG G	CAG Q	GTG V
ATG M	TGG W	CGT R	CTG L	AGC S	AAT N	TTG L	GAT D	TCC S
GCG A	GAC D	CGT R	NCC	AAG K	ACG T	AAG K	GCT A	CAC H
GCT A	AGC S	GGC G	GTT V	TCN	TCT S	AGA R	CCA P	TCG S
AGA R								

TABLE 12 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (C SERIES)

NUCLEOTIDE SEQUENCES

SEQ ID NO:

C.28

TCT	CAC	TCC	TCG	AGT	ACT	ATG	TTG	TGC	314
S	H	S	S	S	T	M	L	C	
TTG	AAC	CCC	CTT	TGC	TGG	ACC	GCG	GCT	
L	N	P	L	C	W	T	A	A	
GGC	AGA	CTC	GAT	ACC	TAC	ACC	AAT	CCC	
G	R	L	D	T	Y	T	N	P	
TCT	ACC	ACG	TCT	AGA	CCA	TCG	AGA		
S	T	T	S	R	P	S	R		

C.29

TCT	CAC	TCC	CCG	AGG	CCG	AAG	GNT	TCT	315
S	H	S	P	R	P	K		S	
GAG	CTT	GAT	TCG	GTT	AAT	TAC	TGG	CCC	
E	L	D	S	V	N	Y	W	P	
GCG	GGG	CGG	GCC	TTC	TTT	CGC	GAC	TTC	
A	G	R	A	F	F	R	D	F	
CCC	ACT	AAN	TTG	GCG	TCT	AGA	CCA	TCG	
P	T		L	A	S	R	P	S	
AGA									
R									

TABLE 13

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (D SERIES)

RELEVANT AMINO ACID SEQUENCES		SEQ ID NO:
D3	HPSSSGGFVVSYRAGGSAAFQNLTQEHPNT SR	316
D6	HSS TWSIDTGR NKPAAVH THTD NNPILSR	317
D7	HSSSTGRVSTLFQVQRAADPSLHRPPARATVSR	318
D12	HSPRLYGGEALFQLL SADDRSPSSSCTKC SR	319
D17	HSSSSYGGRALFA QNPAVY TGHIPS RHTSR	320
D19	HSSSFMDIRKSPVAGTSAEDTVADFADQPRKSR	321
D20	HSSSFVLGTAGGSNVLSAGLALFQQGANGPDSR	322
D21	HSSSVTASGGEVLFKKTAAFSSNRHPSSNAPSR	323
D23	HSSRNLDEVAVGVEEGRGNAFFKKFSTIINSR	324
D25	HSSSFFQFYNNGESRTSADRTPTRSEPDSHRSR	325
D26	HSSSSAFFQVNGRSLSSAGPHLTTNITPPHQSR	326
D29	HSSRNSFFWGDDRVNATAEPPITNMFQHSKRSR	327

SEQUENCES ALIGNED BY APPARENT MOTIFS				SEQ ID NO:
D23	HSSRNLDEVAVGVEEG	**RGNAFFK**	KFSTIINSR	324
D21*	HSSSVTAS	**GGEVLFK**	KTAAFSSNRHPSSNAPSR	323
D3	HPSSSGGFVVSYRA	**GGSAAFQ**	NLTQEHPNT SR	316
D12	HSPRLY	**GGEALFQ**	LL SADDRSPSSSCTKC SR	319
D17	HSSSSY	**GGRALFA**	QNPAVY TGHIPS RHTSR	320
D7	HSSSTG	**RVSTLFQ**	VQRAADPSLHRPPARATVSR	318
D20	HSSSFVLGTAGGSNVLS	**AGLALFQ**	QGANGPDSR	322
D25		**HSSSFFQ**	FYNNGESRTSADRTPTRSEPDSHRSR	325
D26	HS	**SSSAFFQ**	VNGRSLSSAGPHLTTNITPPHQSR	326
D29	HSSRNSFFWGDDRVNATAEP	**PITNMFQ**	HSKRSR	327

D6*	HSS TWSIDTGR NKPAAVH THTD NNPILSR - no apparent motifs	317
D19	HSSSFMDIRKSPVAGTSAEDTVADFADQPRKSR	321

SUBSTITUTE SHEET (RULE 26)

TABLE 13 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (D SERIES)

NUCLEOTIDE SEQUENCES

SEQ. ID. NO.

D.3 328

TCT	CAC	CCC	TCG	AGT	AGT	GGC	GGG	TTC	GTG
S	H	P	S	S	S	G	G	F	V
GTT	TCG	TAT	CGG	GCC	GGG	GGC	TCC	GCG	GCG
V	S	Y	R	A	G	G	S	A	A
TTT	CAG	AAT	CTG	ACT	CAG	GAG	CAT	CCC	AAC
F	Q	N	L	T	Q	E	H	P	N
ACG	GAN	TCT	AGA	CCN	TCG	AGA			
T		S	R	P	S	R			

D.6 329

TCT	CAC	TCC	TCG	NGG	ACT	TGG	TCG	ATT	GAC
S	H	S	S		T	W	S	I	D
ACT	GGG	CGG	ANC	AAC	AAG	CCG	GCC	GCG	GTC
T	G	R		N	K	P	A	A	V
CAT	NAG	ACT	CAT	ACC	GAC	NGG	AAC	AAT	CCC
H		T	H	T	D		N	N	P
ATT	CTG	TCT	AGA	CCA	TCG	AGA			
I	L	S	R	P	S	R			

D.7 330

TCT	CAC	TCC	TCG	AGC	ACT	GGC	AGG	GTT	AGT
S	H	S	S	S	T	G	R	V	S
ACG	CTT	TTT	CAG	GTT	CAG	AGG	GCC	GCG	GAT
T	L	F	Q	V	Q	R	A	A	D
CCT	AGT	CTC	CAC	AGG	CCG	CCG	GCG	CGC	GCC
P	S	L	H	R	P	P	A	R	A
ACC	GTC	TCT	AGA	CCA	TCG	AGA			
T	V	S	R	P	S	R			

D.12 331

TCT	CAC	TCC	CCG	AGG	TTG	TAC	GGC	GGC	GAG
S	H	S	P	R	L	Y	G	G	E
GCG	CTT	TTC	CAG	CTG	CTT	NTG	TCC	GCG	
A	L	F	Q	L	L		S	A	
GAT	GAT	CGC	TCT	CCC	AGC	AGC	AGT	TGC	ACG
D	D	R	S	P	S	S	S	C	T
AAG	TGT	GAN	TCT	AGA	CCA	TCG	AGA		
K	C		S	R	P	S	R		

TABLE 13 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (D SERIES)

NUCLEOTIDE SEQUENCES SEQ. ID NO.

D.17 332

TCT	CAC	TCC	TCG	AGC	TCG	TAC	GGC	GGC
S	H	S	S	S	S	Y	G	G
AGG	GCG	TTG	TTT	GCG	ANC	CAG	AAC	CCC
R	A	L	F	A		Q	N	P
GCG	GTG	TAT	NCG	ACG	GGT	CAC	ATC	CCT
A	V	Y		T	G	H	I	P
TCG	NTT	CGT	CAC	ACG	TCT	AGA	CCA	TCG
S		R	H	T	S	R	P	S
AGA								
R								

D.19 333

CAC	TCC	TCG	AGC	TTT	ATG	GAT	ATC	AGG
H	S	S	S	F	M	D	I	R
AAG	TCC	CCC	GTT	GCC	GGC	TAT	TCC	GCG
K	S	P	V	A	G	Y	S	A
GAG	GAC	ACC	GTT	GCT	GAC	TTC	GCG	GAC
E	D	T	V	A	D	F	A	D
CCC	CGG	AAG	TCT	AGA				
P	R	K	S	R				

D.20 334

CAC	TCC	TCG	AGT	TTT	GTG	CTT	GGG	ACG	GCC
H	S	S	S	F	V	L	G	T	A
GGC	GGT	AGC	AAT	GTG	TTG	TCC	GCG	GGT	CTC
G	G	S	N	V	L	S	A	G	L
GCT	CTC	TTC	CAG	CAG	GGC	GCC	AAT	GGC	CCT
A	L	F	Q	Q	G	A	N	G	P
GAC	TCT	AGA							
D	S	R							

D.21 335

CAC	TCC	TCG	AGC	GTT	ACC	GCC	AGC	GGC	GGC	GAG
S	S	V	T	A	S	G	G	G	G	E
GTG	TTG	TTT	AAG	AAG	ACC	GCG	GCG	TTT		
V	L	F	K	K	T	A	A	F		
AGC	TCC	AAC	CGG	CAT	CCG	AGC	TCT	AAC	GCT	CCG
S	S	N	R	H	P	S	S	N	A	P
TCT	AGA									
S	R									

TABLE 13 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (D SERIES)

NUCLEOTIDE SEQUENCES

SEQ. ID. NO.

D.23

CAC	TCC	TCG	AGG	AAT	TTG	GAC	GAA	GTT	GCA	336
H	S	S	R	N	L	D	E	V	A	
GTA	GGG	GTG	GAG	GAA	GGC	CGC	GGC	AAT	GCC	
V	G	V	E	E	G	R	G	N	A	
TTT	TTC	AAG	AAG	TTT	AGC	ACT	ATC	ATT	AAT	
F	F	K	K	F	S	T	I	I	N	
TCT	AGA									
S	R									

D.25

CAC	TCC	TCG	AGC	TTT	TTT	CAG	TTT	TAC	AAC	337
H	S	S	S	F	F	Q	F	Y	N	
AAT	GGG	GAG	AGC	CGG	ACT	TCC	GCG	GAT	CGT	
N	G	E	S	R	T	S	A	D	R	
ACG	CCC	ACG	AGG	TCC	GAG	CCG	GAT	AGT	CAC	
T	P	T	R	S	E	P	D	S	H	
CGG	TCT	AGA								
R	S	R								

D.26

CAC	TCC	TCG	AGC	TCG	GCC	TTC	TTT	CAG	GTC	338
H	S	S	S	S	A	F	F	Q	V	
AAC	GGG	AGG	AGC	CTG	TCT	TCC	GCG	GGC	CCG	
N	G	R	S	L	S	S	A	G	P	
CAT	CTC	ACC	ACC	AAC	ATC	ACC	CCG	CCC	CAC	
H	L	T	T	N	I	T	P	P	H	
CAG	TCT	AGA								
Q	S	R								

D.29

CAC	TCC	TCG	AGG	AAT	TCT	TTT	TTT	TGG	GGT	339
H	S	S	R	N	S	F	F	W	G	
GAT	GAT	CGG	GTG	AAT	GCA	ACC	GCG	GAG	CCC	
D	D	R	V	N	A	T	A	E	P	
CCT	ATC	ACT	AAT	ATG	TTC	CAG	CAC	TCC	AAG	
P	I	T	N	M	F	Q	H	S	K	
AGG	TCT	AGA								
R	S	R								

TABLE 14

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (AD SERIES)

RELEVANT AMINO ACID SEQUENCES		SEQ ID NO:
AD3	HSSSKYGGMSLFQSQMTAGHHAGTPPYTSRWSR	340
AD14	HSSSLFQSTPGRVKLMPAANDGISSTPGRIPSR	341
AD15	HSSSYNVVAGRAFFRDTAVNTAYPQTAFETRSR	342
AD16	HSSSSSVTVVRANSATSAVKTSNTA LHTDRSR	343
AD17	SHSSRQITAHPLTSVANLRGGDALFTQMRLHSR	344
AD18	HSSSPDSVGGHSFFKSSAGSHHRAHARAPGNSR	345
AD22	HSSSMFQEHRTNLQKNTADRSFTPGYRTDLHSR	346
AD26	HSSSIRTPFSRNYELVSAGASVAPLLLPISTSR	347
AD27	HSSSGSSMFQVDRVVSSADIKMPPVHIRKYDSR	348
AD29	HSSSSLFQRHNRVDMMPAAHNPPKDSATLHGSR	349

SEQUENCES ALIGNED BY APPARENT MOTIFS				SEQ ID NO:
AD15	HSSSYNVV	**AGRAFFR**	DTAVNTAYPQTAFETRSR	342
AD17	SHSSRQITAHPLTSVANLR	**GGDALFT**	QMRLHSR	344
AD18	HSSSPDSV	**GGHSFFK**	SSAGSHHRAHARAPGNSR	345
AD3	HSSSKY	**GGMSLFQ**	SQMTAGHHAGTPPYTSRWSR	340
AD27	HSS	**SGSSMFQ**	VDRVVSSADIKMPPVHIRKYDSR	348
AD22		**HSSSMFQ**	EHRTNLQKNTADRSFTPGYRTDLHSR	346
AD29	H	**SSSSLFQ**	RHNRVDMMPAAHNPPKDSATLHGSR	349
AD14		**HSSSLFQ**	STPGRVKLMPAANDGISSTPGRIPSR	341

AD16 HSSSSSVTVVRANSATSAVKTSNTA LHTDRSR - no apparent motifs 343
AD26 HSSSIRTPFSRNYELVSAGASVAPLLLPISTSR 347

TABLE 14 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (AD SERIES)

NUCLEOTIDE SEQUENCES

AD.3 SEQ ID NO:

TCT S	CAC H	TCC S	TCG S	AGC S	AAG K	TAT Y	GGT G	GGT G	82 ATG M	350
AGT S	TTG L	TTT F	CAG Q	TCG S	CAG Q	ATG M	ACC T	GCG A	GGC G	
CAT H	CAT H	GCG A	GGG G	ACC T	CCC P	CCG P	TAT Y	ACG T	TCC S	
AGG R	TGG W	TCT S	AGA R	CCT P	TCG S	AGA R				

AD.15

TCT S	CAC H	TCC S	TCG S	AGC Y	TAT N	AAC V	GTG V	GTT A	GCT G	351
GGG R	CGC A	TTT F	TTC F	CGG R	GAC D	52 CCC P	GCG A	GTC V	TCC N	
AAC T	ACC A	GCC Y	TAC P	CCT Q	CAG T	ACT A	GCC F	TTC E	GAG T	
ACG R	CGG S	AGA R	CCT P	TCG S	AGA R					

A.16

TCT S	CAC H	TCC S	TCG S	AGT S	TCT S	AGT S	GTG V	ACG T	GTG V	352
GTG V	CGG R	GCG A	AAC N	TCG S	GCT A	52 ACG T	TCC S	GCG A	GTG V	
AAG K	AAG T	ACC S	TCC N	AAC T	ACG A	GCG	NAG L	CTT H	CAT T	
ACG D	GAC R	AGG S	AGA R	CCT P	TCG S	AGA R				

TABLE 14 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (AD SERIES)

NUCLEOTIDE SEQUENCES SEQ ID NO:

AD.18 353

TCT	CAC	TCC	TCG	AGT	CCG	GAT	AGC	GTC	GGG
S	H	S	S	S	P	D	S	V	G
						52			
GGG	CAT	TCG	TTT	TTT	AAG	TCG	TCC	GCG	GGC
G	H	S	F	F	K	S	S	A	G
TCT	CAT	CAC	CGT	GCG	CAT	GCG	CGC	GCG	CCG
S	H	H	R	A	H	A	R	A	P
GGC	AAT	TCT	AGA	CCT	TCG	AGA			
G	N	S	R	P	S	R			

AD.26 354

TCT	CAC	TCC	TCG	AGT	ATT	AGG	ACG	CCT	TTT
S	H	S	S	S	I	R	T	P	F
									52
TCT	CGG	AAT	TAC	GAG	TTG	GTT	TCC	GCG	GGC
S	R	N	Y	E	L	V	S	A	G
GCT	AGC	GTC	GCT	CCT	CTC	CTC	TTG	CCC	ATC
A	S	V	A	P	L	L	L	P	I
TCC	ACT	TCT	AGA	CCT	TCG	AGA			
S	T	S	R	P	S	R			

AD.27 355

TCT	CAC	TCC	TCG	AGT	GGG	AGT	TCG
S	H	S	S	S	G	S	S
	82						
ATG	TTC	TAG	GTG	GAT	CGT	GTC	GTC
M	F	Q	V	D	R	V	V
TCT	TCC	GCG	GAT	ATC	AAG	ATG	CCC
S	S	A	D	K	M	P	P
CCC	GTG	CAC	ATT	CGC	AAG	TAT	GAT
P	V	H	I	R	K	Y	D
TCT	AGA	CCT	TCG	AGA			
S	R	P	S	R			

TABLE 14 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM R26 LIBRARY (AD SERIES)

NUCLEOTIDE SEQUENCES

SEQ ID NO:

AD.29 356

TCT S	CAC H	TCC S	TCG S	AGT S	TCC S	CTG L	TTT F	CAG Q
82 CGT R	CAC H	AAC N	AGG R	GTC V	GAT D	ATG M	ATG M	CCC P
GCG A	GCT A	CAC H	AAC N	CCG P	CCG P	AAG K	GAT D	TCT S
GCC A	ACG T	CTC L	CAC H	GGG G	TCT S	AGA R	CCT P	TCG S
AGA R								

TABLE 15

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM D38 LIBRARY

RELEVANT AMINO ACID SEQUENCES

		SEQ ID NO:
T15-4	HSSRGGGITGVGGGAMFQSRPSVFNAISNNRGHTIPDTFPHTSR	357
T15-10	HSSRAGDSAAGGMALFPDVPLSIRDARPPAHPNSSHLIDWSTSR	358
T15-17	HSSSMFQEGKRRGLPGWICNEGHSHAIHNPNLNQCPDPSPGPSR	359
T15-27	HSSSDMPGRISRGRAMFKEVHATTHADEVGGTNPHHTPSR	360
T15-29	HSSSSAGNCCRGSLFCSCGERTGMDAITPHPHILHRGSSSAASR	361
T15-30	HSSRQLGSNTGEGRTWGTSSQINLDAIPNYTTPHIRQTVPYSSR	362

SEQUENCES ALIGNED BY APPARENT MOTIFS

				SEQ ID NO:
T15-29	HSSSSAGNC	**CRGSLFC**	SCGERTGMDAITPHPHILHRGSSSAASR	361
T15-10	HSSRAGDSAA	**GGMALFP**	DVPLSIRDARPPAHPNSSHLIDWSTSR	358
T15-4	HSSRGGGITGV	**GGGAMFQ**	SRPSVFNAISNNRGHTIPDTFPHTSR	357
T15-27	HSSSDMPGRIS	**RGRAMFK**	EVHATTHADEVGGTNPHHTPSR	360
T15-17		**HSSSMFQ**	EGKRRGLPGWICNEGHSHAIHNPNLNQCPDPSPGP	359

T15-30 HSSRQLGSNTGEGRTWGTSSQINLDAIPNYTTPHIRQTVPYSSR 362

- no apparent motif

TABLE 15 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM D38 LIBRARY

NUCLEOTIDE SEQUENCES

T.15-4 SEQ ID NO: 363

CAC H	TCC S	TCG S	AGG R	GGT G	GGG G	GGC G	ATC I	ACC T	121 GGG G
GTC V	GGT G	GGG G	GGT G	GCG A	ATG M	TTT F	CAG Q	TCC S	91 CGT R
CCC P	TCT S	GTT V	TTC F	AAC N	GCC A	ATT I	AGC S	AAC N	61 AAT N
CGC R	GGC G	CAC H	ACG T	ATT I	CCC P	GAC D	ACT T	TTT F	31 CCC P
CAC H	ACT T	TCT S	AGA R	ATC I	GAA E	GGT G	CGC R	GCT A	1 AGA R

T.15-10 364

CAC H	TCC S	TCG S	AGA R	GCG A	GGG G	GAC D	AGT S
GCT A	121 GCG A	GGC G	GGC G	ATG M	GCG A	CTT L	TTT F
CGC P	GAT D	GTC V	91 CCG P	CTG L	TCG S	ATT I	CGT R
GAC D	61 GCC A	AGG R	CCC P	CCT P	GCC A	CAC H	CCT P
AAT N	AGC S	AGC S	CAT H	CTT L	ATC I	GAT D	TGG W
AGC S	ACT T	TCT S	AGA R	ATC I	GAA E	GGT G	CGC R
GCT A	AGA R						

TABLE 15 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM D38 LIBRARY

NUCLEOTIDE SEQUENCES SEQ ID NO:

T.15-17 365

CAC H 121	TCC S	TCG S	AGC S	ATG M	TTC F	CAG Q	GAG E	GGT G
AAG K	CGG R 91	AGG R	GGT G	TTG L	CCG P	GGT G	TGG W	ATC I
TGC C	AAT N	GAG E 61	GGC G	CAT H	TCT S	CAC H	GCC A	ATC I
CAC H	AAT N	CCC P	AAT N 31	CTC L	AAC N	CAG Q	TGT C	CCC P
GAC D	CCG P	AGT S	CCG P	GGC G	CCT P	TCT S	AGA R	ATC I
GAA E	GGT G	CGC R	GCT A	AGA R				

T.15-27 366

CAC H	TCC S	TCG S	AGT S	GAC D	ATG M	CCG P	GGG G	CGG R 79	ATT I	TCT S
CGG R	GGT G	CGC R	GCC A	ATG M	TTC F	AAG K	GAG E	GTT V	CAC H	GCC A
ACT T	ACC T	CAT H	GCC A	GAT D	GAG E	GTG V	GGC G	GGC G	ACG T	AAC N
CCT P	CAT H	CAT H	ACC T	CCG P	TCT S	AGA R				

TABLE 15 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM D38 LIBRARY

NUCLEOTIDE SEQUENCES SEQ ID NO:

T.15-29 367

									121
CAC	TCC	TCG	AGT	TCG	GCG	GGC	AAC	TGT	TGC
H	S	S	S	S	A	G	N	C	C
									91
CGG	GGT	AGC	CTT	TTC	TGC	TCT	TGC	GGT	GAG
R	G	S	L	F	C	S	C	G	E
									61
CGT	ACT	GGT	ATG	GAC	GCC	ATC	ACC	CCC	CAT
R	T	G	M	D	A	I	T	P	H
									31
CCG	CAT	ATC	CTC	CAC	CGC	GGG	AGC	TCC	TCT
P	H	I	L	H	R	G	S	S	S
GCC	GCC	TCT	AGA	ATC	GAA	GGT	CGC	GCT	AGA
A	A	S	R	I	E	G	R	A	R

T.15-30 368

									121
CAC	TCC	TCG	AGG	CAG	CTG	GGT	TCG	AAT	ACG
H	S	S	R	Q	L	G	S	N	T
									91
GGG	GAG	GGT	CGG	ACT	TGG	GGT	ACT	TCC	TCG
G	E	G	R	T	W	G	T	S	S
									61
CAG	ATC	AAC	CTG	GAC	GCC	ATC	CCT	AAC	TAC
Q	I	N	L	D	A	I	P	N	Y
									31
ACC	ACC	CCC	CAC	ATT	CGG	CAG	ACG	GTT	CCG
T	T	P	H	I	R	Q	T	V	P
									1
TAC	TCC	TCT	AGA	ATC	GAA	GGT	CGC	GCT	AGA
Y	S	S	R	I	E	G	R	A	R

TABLE 16

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM DC43 LIBRARY

AMINO ACID SEQUENCES

		SEQ ID NO:
T20-1	HSSSNYGGADRAWGGRSLFTSAVTGCGNSPRNDRDERRPNTRTSNVTSR	369
T20-5	HSSRTAKEGCSGGASLFLELRAQCGCGAHRNTPPSHCLPVETKNCDDSR	370
T20-13	HSSSINDSGSRTWSGGCGISRDGARALFLDDPSRDPLSR	371

SEQUENCES ALIGNED BY APPARENT MOTIFS

				SEQ ID NO:
T20-1	HSSSNYGGADRAW	**GGRSLFT**	SAVTGCGNSPRNDRDERRPNTRTSNVTSR	369
T20-5	HSSRTAKEGCS	**GGASLFL**	ELRAQCGCGAHRNTPPSHCLPVETKNCDDSR	370
T20-13	HSSSINDSGSRTWSGGCGISRD	**GARALFL**	DDPSRDPLSR	371

TABLE 16 (con'd)

POLYCLONAL ANTI-FERRITIN BINDER SEQUENCES
FROM DC43 LIBRARY

<u>NUCLEOTIDE SEQUENCES</u>

<u>SEQ ID NO:</u>

T.20-1

372

CAC TCC TCG AGC AAT TAC GGT GGC GCG GAT
H S S S N Y G G A D

AGG GCG TGG GGT GGG CGG TCG CTG TTC ACG
R A W G G R S L F T

AGC GCT GTG ACC GGT TGT GGT AAC TCC CCC
S A V T G C G N S P

CGT AAC GAT AGG GAC GAG CGC CGT CCT AAC
R N D R D E R R P N

ACG AGG ACT AGT AAT GTT ACC TCT AGA ATC
T R T S N V T S R I

GAA GGT CGC GCT AGA
E G R A R

T.20-5

373

CAC TCC TCG AGA ACG GCT AAG GAG GGG AGT GTG
H S S R T A K E G S V

GGC GGG GCC AGC CTG TTT TTG GAG CTT AGG GCC
G G A S L F L E L R A

CAG TGT GGT TGT GGT GCT CAC CGT AAC ACC CCG
Q C G C G A H R N T P

CCG TCG CAC TGC TTG CCT GTT GAG ACA AAG AAT
P S H C L P V E T K N

TGT GAT GAC TCT AGA ATC GAA GGT CGC GCT AGA
C D D S R I E G R A R

T.20-13

374

CAC TCC TCG AGT ATT AAT GAC AGT GGT AGC AGG
H S S S I N D S G S R

ACG TGG TCG GGT GGT TGT GGT ATC TCC
T W S G G C G I S

CGG GAT GGC GCC CGC CGC GCC CTT TTC CTG GAC
R D G A R R A L F L D

GAT CCC TGC CGC GAC CCT TTG TCT AGA
D P C R D P L S R

ATC GAA GGT CGC GCT AGA
I E G R A R

WHAT IS CLAIMED IS:

1. A method of determining the presence or absence of an analyte of interest in a sample by an affinity assay comprising:

(a) providing (i) a labeled conjugate comprising at least one label attached to a functional surrogate capable of competing effectively with an analyte of interest for a limiting amount of an affinity receptor for said analyte, said labeled conjugate exhibiting an activity that is altered on interaction of said labeled conjugate with said affinity receptor and which activity can be measured and related to the presence or absence of said analyte in a given sample, and (ii) said affinity receptor;

(b) combining said labeled conjugate and affinity receptor with a sample suspected of containing said analyte to provide a measure of said activity;

(c) measuring said activity; and

(d) relating said activity to the presence or absence of said analyte in said sample.

2. The method of claim 1 in which said interaction is a binding interaction.

3. The method of claim 1 in which said functional surrogate is further characterized as exhibiting a competitive binding profile that is substantially similar to that exhibited by said analyte for said affinity receptor.

4. The method of claim 1 in which said functional surrogate is further characterized as exhibiting a selective binding affinity (K_a) for said affinity receptor which is substantially similar to that exhibited by said analyte.

5. The method of claim 1 in which said relating step provides a quantitative measure.

6. The method of claim 1 in which said functional surrogate is obtained by screening a random peptide library with an affinity receptor of said analyte.

7. The method of claim 6 in which said random peptide library comprises a plurality of peptides whose structures are not dictated by the primary sequence of said analyte.

8. The method of claim 1 in which the molecular structure of said functional surrogate corresponds to an epitope of said analyte.

9. The method of claim 8 in which the structure of said epitope was previously unknown.

10. The method of claim 1 in which the molecular structure of said functional surrogate differs from that of a known epitope of said analyte.

11. The method of claim 10 in which said molecular structure does not include a primary sequence of eight or more consecutive amino acid residues which can be found along the naturally occurring sequence of said analyte.

12. The method of claim 1 in which said functional surrogate has a molecular weight of 2000 daltons or less.

13. The method of claim 1 in which said functional surrogate comprises a peptide.

14. The method of claim 1 in which said analyte is a hapten.

15. The method of claim 1 in which said analyte is an antigen.

16. The method of claim 1 in which said analyte is an antibody.

17. The method of claim 1 in which said combining step comprises forming an affinity receptor-labeled conjugate complex.

18. The method of claim 17 in which said combining step further comprises displacing said labeled conjugate from said complex with said analyte.

19. The method of claim 1 in which said combining step comprises providing competition among said analyte and said labeled conjugate for said affinity receptor.

20. The method of claim 1 in which said combining step comprises forming an affinity receptor-analyte complex.

21. The method of claim 20 in which said combining step further comprises forming an affinity receptor-labeled conjugate complex.

22. The method of claim 1 in which said sample is obtained from a biological fluid selected from the group consisting of urine, semen, saliva, sweat, blood, serum, plasma, cerebrospinal fluid, tears, vaginal or nasal fluids.

23. The method of claim 1 in which said sample is obtained from a cell-free extract.

24. The method of claim 1 in which said label is selected from the group consisting of a chromogenic agent, UV absorber, fluorescent molecule, a chemiluminescent compound, an enzyme, an enzyme fragment, an enzyme substrate or a group having the potential for exhibiting at least one of the above-recited activities.

25. The method of claim 24 in which said label comprises an enzyme.

26. The method of claim 25 in which said enzyme exhibits glucose-6-phosphate dehydrogenase (G6PDH) activity.

27. The method of claim 1 in which said combining step comprises (i) mixing said affinity receptor and sample, and (ii) adding said labeled conjugate to the resulting mixture.

28. The method of claim 1 in which said activity is measured as a rate of change.

29. The method of claim 1 in which said analyte is a polypeptide, a polysaccharide, a polynucleotide, a glycoprotein or a lipid-containing macromolecule.

30. The method of claim 1 in which said analyte is a fertility/pregnancy-related hormone, is related to an infectious disease, is a cardiac marker or a tumor marker.

31. The method of claim 1 in which said analyte is associated with a bacterial or viral infectious agent.

32. The method of claim 1 in which said analyte is ferritin, follicle stimulating hormone (FSH), thyroid-stimulating hormone (TSH), human growth hormone (hGH), immunoglobulin E (IgE), prolactin, parathyroid hormone (PTH), human placental lactogen (HPL), human chorionic gonadotropin (hCG), human leutinizing hormone (hLH), cytomegalovirus (CMV), chlamydia, streptococcus A, rubella, toxoplasma, herpes, hepatitis, CK-MB, myoglobin, myosin light chain, troponin, carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), prostrate specific antigen (PSA), CA125 (a tumor marker).

33. The method of claim 1 in which said analyte is an allergen.

34. The method of claim 1 in which said analyte has a molecular weight in excess of about 100,000 daltons.

35. A method of determining the presence or absence of an analyte of interest in a sample by an homogeneous enzyme affinity assay comprising:

(a) providing (i) an enzyme conjugate comprising an enzyme attached to at least one functional surrogate of an analyte of interest, said functional surrogate capable of competing effectively with said analyte for a limiting amount of an affinity receptor for said analyte, said enzyme conjugate exhibiting an activity that is altered on interaction of said enzyme conjugate to said affinity receptor and which activity can be measured and related to the amount of said analyte present in a given sample; (ii) said affinity receptor, and (iii) a substrate for said enzyme;

(b) combining said enzyme conjugate, affinity receptor, and enzyme substrate with a sample suspected of containing said analyte to provide a measure of said enzyme activity;

(c) measuring said enzyme activity; and

(d) relating said enzyme activity to the presence or absence of said analyte in said sample.

36. A method of determining the presence or absence of an analyte of interest in a sample by an homogeneous fluorescence polarization affinity assay comprising:

(a) providing (i) a labeled conjugate comprising a fluorescent material attached to a functional surrogate of an analyte of interest, said functional surrogate capable of competing effectively with an analyte of interest for a limiting amount of an affinity receptor for said analyte, said labeled conjugate exhibiting an activity that is altered on interaction of said labeled conjugate with said affinity receptor and which activity can be measured and related to the presence or absence of said analyte in a given sample, and (ii) said affinity receptor;

(b) combining said labeled conjugate and affinity receptor with a sample suspected of containing said analyte to provide a measure of said activity;

(c) measuring said activity; and

(d) relating said activity to the presence or absence of said analyte in said sample.

37. A method of determining the presence or absence of an analyte of interest in a sample by an homogeneous cloned enzyme

donor affinity assay comprising:

(a) providing (i) a labeled conjugate comprising an enzyme donor fragment attached to a functional surrogate of an analyte of interest, said functional surrogate capable of competing effectively with said analyte for a limiting amount of an affinity receptor for said analyte, said labeled conjugate, on interaction with an enzyme acceptor fragment, exhibiting an activity that is altered in the presence of said affinity receptor and which activity can be measured and related to the amount of said analyte present in a given sample, (ii) said enzyme acceptor fragment, and (iii) said affinity receptor;

(b) combining said labeled conjugate, enzyme acceptor fragment, and affinity receptor with a sample suspected of containing said analyte to provide a measure of said activity;

(c) measuring said activity; and

(d) relating said activity to the presence or absence of said analyte in said sample.

38. An affinity assay kit comprising:

(a) a labeled conjugate diposed in a first container means, said labeled conjugate comprising at least one label attached

to a functional surrogate of an analyte of interest, said functional surrogate capable of competing effectively with said analyte for a limiting amount of an affinity receptor for said analyte, said labeled conjugate capable of exhibiting an activity that is altered on interaction of said labeled conjugate with said affinity receptor and which activity can be measured and related to the amount of said analyte present in a given sample; and

(b) disposed in a second container means said affinity receptor and, optionally, any substance required for said labeled conjugate to exhibit said activity.

39. The kit of claim 38 in which said substance comprises an enzyme substrate.

40. The kit of claim 39 in which said substance comprises an enzyme acceptor fragment.

41. The kit of claim 38 in which said activity increases on binding of said labeled conjugate with said affinity receptor.

42. The kit of claim 38 in which said activity decreases on binding of said labeled conjugate with said affinity receptor.

43. The kit of claim 38 in which said analyte is selected from the group consisting of ferritin, a hepatitis antigen, an antibody against a hepatitis antigen, human chorionic gonadotropin (hCG), human leutinizing hormone (hLH), cytomegalovirus (CMV), chlamydia, streptococcus A, rubella, toxoplasma, herpes, hepatitis, CK-MB, myoglobin, myosin light chain, troponin, carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), prostrate specific antigen (PSA), CA125 (a tumor marker).

44. A functional surrogate of an analyte of interest comprising a peptide having an immunoreactive group that allows said surrogate to compete effectively with said analyte for a limiting amount of an affinity receptor for said analyte.

45. The functional surrogate of claim 44 in which said peptide comprises about 4 to about 100 amino acid residues.

46. The functional surrogate of claim 44 which competes effectively with ferritin for a limiting amount of an affinity receptor for ferritin.

47 . The functional surrogate of claim 44 which competes effectively with a hepatitis antigen for a limiting amount of an affinity receptor for said hepatitis antigen.

48. The functional surrogate of claim 44 in which said antigen is the hepatitis A antigen.

49. The functional surrogate of claim 44 in which said antigen is the hepatitis B antigen.

50. The functional surrogate of claim 44 in which said antigen is the hepatitis C antigen.

51. A functional surrogate comprising a peptide having up to about 35 amino acid residues, including the primary sequence motifs depicted in SEQ. ID. NOS. 1-89, 105-115, 127-134, 137-154, 169-180, 193-203, 215-226, 239-247, 255-260, or 267-269.

52. The functional surrogate of claim 51 which further includes 2-10 amino acid residues flanking said sequence motifs.

53. The functional surrogate of claim 52 in which said flanking residues are selected among those depicted in said SEQ. ID. NOS.

54. A labeled conjugate comprising at least one label attached to a functional surrogate of an analyte of interest, said functional surrogate capable of competing effectively with said analyte for a limiting amount of an affinity receptor for said analyte, said labeled conjugate capable of exhibiting an activity that is altered on interaction of said labeled conjugate with said affinity receptor and which activity can be measured and related to the amount of said analyte present in a given sample.

55. A recombinant DNA construct comprising a DNA sequence encoding a functional surrogate of an analyte of interest, said functional surrogate capable of competing effectively with said analyte for a limiting amount of an affinity receptor for said analyte.

56. The construct of claim 53 in which said DNA sequence is selected from at least those sequences depicted in SEQ. ID. NOS. 90-104, 116-126, 135-136, 155-168, 181-192, 204-214, 227-238, 248-254, 261-266, or 270-272, which encode a primary sequence motif.

57. A transforming vector including the construct of claim 55.

58. The vector of claim 57 which is autonomously replicating.

59. Bacteriophage transformed by the vector of claim 57.

60. A microorganism transformed by the vector of claim 57.

61. A microorganism infected with the bacteriophage of claim 59.

62. A method of obtaining a functional surrogate of an analyte of interest comprising:

(a) selecting an affinity receptor exhibiting a selective affinity for an analyte of interest;

(b) screening a random peptide library with said affinity receptor for a binding peptide;

(c) isolating said binding peptide and identifying its structure.

63. The method of claim 62 which further comprises synthesizing said peptide and verifying its capacity to compete with said analyte for a limiting amount of said affinity receptor.

64. The method of claim 62 which further comprises conjugating said peptide to at least one label.

65. The method of claim 62 in which said library is a phage display random peptide library.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10498

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :Please See Extra Sheet
US CL :Please See Extra Sheet
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : Please See Extra Sheet

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, Medline, Aidsline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	J.P. RANSOM, "Practical competitive binding assay methods" published 1976 by The C.V. Mosby Company, (Saint Louis) page 2, see entire document.	1-5, 8-21, 23-25, 29-33, and 35 -------- 6, 7, 22, 26-28, 34, 36-52, and 54-56
X --- Y	CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY, Volumne 75, Number 1, issued April 1995, Dybwad et al, "Structural Characterization of Peptides That Bind Synovial Fluid Antibodies From RA Patients: A Novel Strategy for Identification of Disease-Related Epitopes Using a Random Peptide Library", pages 45-50, see entire document.	6, 7, 55-65 --------- 44-52 and 54

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 04 SEPTEMBER 1996	Date of mailing of the international search report 02 OCT 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY STUCKER Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10498

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	GENE, Volume 146, issued 1994, Motti et al, "Recognition by Human Sera and Immunogenicity of HBsAg Mimotopes Selected From an M13 Phage Display Library (Hepatitis B virus surface antigen; affinity selection; immuno-screening; serum antibodies; immunization; vaccines; diagnostics) ", pages 191-198, see entire document.	6, 7, 44, 49, 55-65 ------- 45-52 and 54

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10498

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely

2. [X] Claims Nos.: 53
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

because the sequence numbers were not put in the claim after "SEQ. ID. NOS."

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [X] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-43; 44-52,54 (first 10 amino acid sequences); 55-61 (first 10 DNA sequences); 62-65

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
[ ] The additional search fees were accompanied by the applicant's protest.
[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10498

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

G01N 33/53, 33/573, 33/537; C07K 7/00, 14/00; C12N 15/09; C12Q 1/70, 1/32

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/5, 7.1, 7.2, 7.4, 7.9, 7.91, 7.92, 26, 320.1, 974, 975; 436/813, 814, 818; 530/324, 325, 326, 327, 328, 329, 330, 350; 935/12, 23, 57, 58, 70, 72

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/5, 7.1, 7.2, 7.4, 7.9, 7.91, 7.92, 26, 320.1, 974, 975; 436/813, 814, 818; 530/324, 325, 326, 327, 328, 329, 330, 350; 935/12, 23, 57, 58, 70, 72